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(54) Title: PRODUCER CELL FOR THE PRODUCTION OF RETROVIRAL VECTORS

(57) Abstract: A method is provided for modifying a producer cell which producer cell comprises integrated into its genome a provirus which provirus comprises one or more recombinase recognition sequences within or upstream of its 3' LTR, the method comprising: introducing into the cell a construct comprising a 5' recombinase recognition sequence, an LTR and a 3' recombinase recognition sequence in that order, in the presence of a recombinase which is capable of acting on the recombinase recognition site(s) such that the nucleotide sequence between the 5' and 3' recombinase recognition sequences in the construct is introduced into the provirus.

PRODUCER CELL FOR THE PRODUCTION OF RETROVIRAL VECTORS

FIELD OF THE INVENTION

The present invention relates to retroviral vectors, in particular to high titre regulatable retroviral vectors.

BACKGROUND TO THE INVENTION

Retroviruses have been proposed as a delivery system (otherwise expressed as a delivery vehicle or delivery vector) for *inter alia* the transfer of a nucleotide sequence of interest (NOI), or a plurality of NOIs, to one or more sites of interest. The transfer can occur *in vitro, ex vivo, in vivo,* or combinations thereof. When used in this fashion, the retroviruses are typically called retroviral vectors or recombinant retroviral vectors. Retroviral vectors have been exploited to study various aspects of the retrovirus life cycle, including receptor usage, reverse transcription and RNA packaging (reviewed by Miller, 1992 Curr Top Microbiol Immunol 158:1-24).

In a typical recombinant retroviral vector for use in gene therapy, at least part of one or more of the *gag, pol* and *env* protein coding regions may be removed from the virus. This makes the retroviral vector replication-defective. The removed portions may even be replaced by a NOI in order to generate a virus capable of integrating its genome into a host genome but wherein the modified viral genome is unable to propagate itself due to a lack of structural proteins. When integrated in the host genome, expression of the NOI occurs - resulting in, for example, a therapeutic effect. Thus, the transfer of a NOI into a site of interest is typically achieved by: integrating the NOI into the recombinant viral vector; packaging the modified viral vector into a virion coat; and allowing transduction of a site of interest - such as a targetted cell or a targetted cell population.

It is possible to propagate and isolate quantities of retroviral vectors (e.g. to prepare suitable titres of the retroviral vector) for subsequent transduction of, for example, a site of interest by using a combination of a packaging or helper cell line and a recombinant vector.

In some instances, propagation and isolation may entail isolation of the retroviral *gag, pol* and *env* genes and their separate introduction into a host cell to produce a "packaging cell line". The packaging cell line produces the proteins required for packaging retroviral RNA but it does not produce RNA-containing retroviral vectors. However, when a recombinant vector carrying a NOI and a *psi* region is introduced into the packaging cell line, the helper proteins can package the *psi*-positive recombinant vector to produce the recombinant virus stock. This can be used to infect cells to introduce the NOI into the genome of the cells. The recombinant virus whose genome lacks all genes required to make viral proteins can infect only once and cannot propagate. Hence, the NOI is introduced into the host cell genome without the generation of potentially harmful retrovirus. A summary of the available packaging lines is presented in "Retroviruses" (1997 Cold Spring Harbour Laboratory Press Eds: JM Coffin, SM Hughes, HE Varmus pp 449).

Retroviral packaging cell lines have been developed to produce retroviral vectors. These cell lines are designed to express three components, which may be located on three separate expression constructs. The *gag/pol* expression construct encodes structural and enzymatic components required in particle formation, maturation, reverse transcription and integration. The envelope (*env*) construct expresses a retroviral or non-retroviral envelope protein, which mediates viral entry into cells by binding to its cognate receptor. The third expression construct produces the retroviral RNA genome containing a *psi* region, which is packaged into mature, enveloped retroviral particles.

It has been observed that different methods, such as electroporation, transfection and retroviral transduction, which have been used to introduce the retroviral expression construct for the RNA genome, termed "the genome", into packaging cells produce different results. These different results can include different end points or "yield" of retroviral producer lines resulting from the derived cell lines. Moreover, electroporation and transfection methods can be problematic in the sense that the titre levels are not always at a satisfactory level.

By way of example, the transfection of a plasmid DNA construct into packaging cells from a MLV packaging cell line of human origin, called FLYA13, yielded low retroviral

vector titres even when different transfection reagents such as calcium phosphate precipitation and fugene transfection reagent were used. The average titres from selected stably transfected cell lines clones ranged from about 10^3 to about 10^4 per ml. In addition, clones generated by electroporation of constructs gave similar titres of from about 10^3 to about 10^4 per ml with no clones identified producing at $>10^5$ per ml. However, when MLV vector particles are prepared in a transient transfection system with a different envelope pseudotype to the packaging cell, and used to transduce a retroviral packaging cell, stably transduced cell lines made by this transduction method produce retrovirus at 10^6 to 10^7 per ml. Therefore, these results suggest that retroviral transduction is a preferred method for genome introduction into packaging cell lines in order to generate high titre producer cell lines. However, when retroviral transduction is used to introduce a regulated/inactivated retroviral vector genome into packaging cell lines, the regulated retroviral vectors may not be produced in sufficient quantities from these cell lines.

By way of example, some retroviral vectors may comprise (i) internal expression constructs which are themselves regulated or (ii) regulated elements which are present in retroviral 3' LTR sequences, either by design or by their nature. Examples of these regulated vectors include but are not limited to hypoxic regulated vectors and self inactivating (SIN) vectors. If transduced producer cell lines are generated with these regulated vectors, the regulated or inactivated 3' U3 sequence of the LTR is copied to the 5' LTR by the process of retroviral reverse transcription and integration. Therefore, in the producer cell line, the 5' U3 promoter sequence directing expression of retroviral RNA genomes is identical to the regulated or inactivated 3' U3 promoter. This will result in very low levels of retroviral genome production and consequently low titres of functional retrovirus vectors being produced.

One example of such a regulated retroviral system includes MLV and lentivirus vector constructs where the 3' retroviral U3 enhancer element is replaced with a hypoxia responsive element (HRE) or other physiologically regulated, tumour specific or tissue-specific promoters. When these vectors are used to make a transduced producer cell line, the 3' U3 sequence containing the HRE element is copied to the 5' LTR position and retroviral genomes will only be produced under hypoxic conditions or chemical mimics

of hypoxia, such as heavy metal ions and desferrioxamine. Such a requirement for "induction for retroviral production" is not preferable as the different hypoxia induction protocols negatively affect retroviral producer cell viability.

By way of further example, lentivector U3 enhancers are dependent on the transactivator TAT for transcriptional activation. Therefore, a lentivector producer cell line generated by transduction requires the presence of TAT for high level expression of the lentivector genome construct. The expression of TAT is not preferable in such a packaging cell line and therefore, in the absence of TAT, only very low titres will be produced from transduced producer cells generated in this way.

Another example of a regulated retroviral systems includes MLV or lentivirus self-inactivating (SIN) vectors. These vectors contain deletions of the elements in their 3' U3 sequences responsible for transcriptional activity. Therefore, on transduction of target cells, the transcriptionally inactive 3' U3 sequence is copied to the 5' LTR position. In standard configurations, an internal expression cassette directs therapeutic or marker gene expression. However, if SIN vectors are used to make a transduced retroviral producer line, there will be no transcriptional elements present to direct high levels of retroviral RNA genome expression.

Although it is possible to carry out retroviral transduction with much lower-titre vector stocks, for practical reasons, high-titre retrovirus is desirable, especially when a large number of cells must be infected. In addition, high titres are a requirement for transduction of a large percentage of certain cell types. For example, the frequency of human hematopoietic progenitor cell infection is strongly dependent on vector titre, and useful frequencies of infection occur only with very high-titre stocks (Hock and Miller 1986 Nature 320: 275-277; Hogge and Humphries 1987 Blood 69: 611-617). In these cases, it is not sufficient simply to expose the cells to a larger volume of virus to compensate for a low virus titre. On the contrary, in some cases, the concentration of infectious vector virions may be critical to promote efficient transduction.

SUMMARY OF THE INVENTION

We have now shown that it is possible to obtain transduced producer cells capable of producing high titre regulated retroviral vectors by replacing at least the 3'LTR of the integrated provirus using a recombinase based system. Thus whereas with the prior art, the U3 region of the 3'LTR is the same as that of the U3 region of the 5' LTR (and vice versa for the U5 region) in the provirus due to the way in which the viral vector integrates, the introduction of, for example, a replacement 3'LTR results in a provirus that has a 5'LTR and a 3'LTR that differ. The packaged viral vectors produced by transcription of the proviral genome within the producer cells may then ultimately be used to transduce target cells where the regulatable sequences present in the 3'LTR of the provirus in the producer cells are then present in the 5'LTR of the provirus in the target cells and consequently regulate transcription from the provirus as required.

This allows the introduction of a 3'LTR, for example a regulatable 3'LTR, into the provirus that was not desirable in the original viral vector used to transduce the producer cells since the consequential appearance of the regulatable 3'LTR U3 sequences in the 5'LTR in the provirus may lead to a reduced viral titre.

Consequently, the present invention allows transduced producer cells to be constructed that are capable of producing high titre regulated retroviral vectors by virtue of comprising a 5'LTR that directs high level expression of the viral genome in the producer cell and a different 3'LTR which as a result of the process of integration into a target cell will then result in a provirus in the target cell genome that exhibits regulatable expression.

In particular, the present invention allows the modification of a provirus integrated into the genome of the producer cells that have been selected for their high titre virus production such that the resulting packaged viral particles produced from the provirus may be used to transduce target cells resulting in a provirus integrated into the genome of the target cells that has a different, and preferably regulatable 5'LTR to that of the producer cell provirus.

The present invention is not limited to replacement of the 3'LTR of the provirus in the high titre producer cells, but may also include replacement of the 5'LTR and other viral sequences and/or the introduction of NOIs by the use of suitable constructs, as shown in the Figures.

Accordingly, the present invention provides a method of modifying a producer cell which producer cell comprises integrated into its genome a provirus which provirus comprises one or more recombinase recognition sequences within or upstream of its 3' LTR, the method comprising: introducing into the cell a construct comprising a 5' recombinase recognition sequence, an LTR and a 3' recombinase recognition sequence in that order, in the presence of a recombinase which is capable of acting on the recombinase recognition site(s) such that the nucleotide sequence between the 5' and 3' recombinase recognition sequences in the construct is introduced into the provirus.

Preferably the LTR is a heterologous regulatable LTR.

The present invention further provides a nucleic acid vector comprising a 5' recombinase recognition sequence, a regulatable LTR and a 3' recombinase recognition sequence in that order.

In any of the above aspects and embodiments of the invention, preferably the construct, nucleic acid molecule and/or nucleic acid vector further comprises at least one NOI between the 5' recombinase recognition sequence and the regulatable LTR.

Preferably the construct, nucleic acid molecule and/or nucleic acid vector further comprises a 5'LTR and/or a packaging signal

In one embodiment of the invention, the LTR is inactive/transcriptionally quiescent.

The construct, nucleic acid molecule and/or nucleic acid vector of the invention may be used in a recombinase assisted method to introduce a regulated LTR into a proviral genome integrated into a producer cell genome.

The present invention also provides a producer cell obtainable by the method of the invention, preferably a high titre producer cells. Also provided is an infectious retroviral particle obtained by the above method.

The present invention further provides a high titre producer cell comprising integrated into its genome a provirus, which provirus comprises a recombinase recognition site, a 5' LTR and a 3'LTR which 3'LTR differs from the 5'LTR. Such a producer cell will typically have been produced by the method of the invention.

Preferably the 5'LTR and the 3'LTR referred to for the purposes of comparison are both "active". The term "active" within the present context means transcriptionally active, that is to say, the 5'LTR comprises a promoter that directs transcription of the viral genome and the 3'LTR comprises a transcriptional stop sequence to terminate transcription. This distinction is relevant since if a provirus produced by the method of the invention comprises more than one 5' LTR or 3'LTR, at least one but not all must be active to allow viral production. Further, if the provirus comprises more than one 3'LTR then it is generally the upstream one that will be active since transcription will tend not to read through to the downstream 3' LTR.

In addition, where the method of the invention results in an insertion of a 3'LTR upstream of the original 3'LTR, the comparison should be performed between the additional 3'LTR and the original 5'LTR and not the two original LTRs. Thus it is permitted to have a 5'LTR and 3'LTR within the same provirus that are the same provided that there is also a 5'LTR and 3'LTR that differ.

In another aspect, the present invention provides a derived producer cell comprising integrated into its genome a retroviral vector comprising in the 5' to 3' direction a first 5' LTR; a second NOI operably linked to a second regulatable 3' LTR; and a third 3'LTR; wherein the third 3'LTR is positioned downstream of the second regulatable 3'LTR in the producer cell.

Preferably the first 5' LTR comprising 5'R and 5' U5 sequences is derivable from a first vector; the second NOI operably linked to a second regulatable 3' LTR is derivable from a second vector; and the third 3'LTR is derivable from the first vector.

In a preferred embodiment, the first vector further comprises an internal LTR located upstream of the first NOI and downstream of the packaging signal wherein the internal LTR comprises a heterologous U3 sequence linked to heterologous R and U5 sequences.

Preferably the heterologous R and U5 sequences are lentiviral derivable R and U5 sequences, such as EIAV R and U5 sequences.

In a further preferred embodiment, the third 3'LTR is transcriptionally active but expression is directed away from the second regulatable 3'LTR.

In another embodiment, the second vector comprises a second NOI operably linked to a second regulatable 3'LTR comprising at least one recombinase recognition sequence. Preferably the second regulatable 3'LTR comprises a deletion in the U3 sequences in the 3'LTR.

Preferably, the second NOI comprises a discistronic construct, more preferably a discistronic construct comprising a therapeutic gene, an internal ribosomal entry site (IRES) and a reporter gene.

The present invention further provides in another embodiment, a method for producing a high titre regulatable retroviral vector, the method comprising the steps of:

(i) providing a derived producer cell comprising integrated into its genome a first vector;

(ii) introducing a second vector into the derived producer cell using a recombinase assisted method; wherein the derived producer cell comprises a retroviral vector comprising in the 5' to 3' direction a first 5' LTR; a second NOI operably linked to a second regulatable 3' LTR; and a third 3'LTR; wherein the third 3'LTR is positioned downstream of the second regulatable 3'LTR in the derived producer cell.

The present invention also provides the use of a recombinase assisted mechanism to introduce a regulated 3'LTR into a derived producer cell line to produce a high titre regulated retroviral vector.

Aspects of the present invention are also presented in the accompanying claims and in the following description and discussion.

These aspects are presented under separate section headings. However, it is to be understood that the teachings under each section heading are not necessarily limited to that particular section heading.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is advantageous because:

(i) it enables regulated retroviral vectors to be produced at high titres from transduced producer cell lines.

(ii) it removes the uncertainty associated with the process of producer cell line derivation and the necessity to screen large numbers of producer cell lines each time a new retroviral expression construct is introduced into a producer cell line.

(iii) it greatly facilitates the generation of high titre retroviral stocks without the use of marker genes (such as but not limited to β-galactosidase, green fluorescent protein) and antibiotic resistance genes.

(iv) it avoids the derivation of low titre transfected producer cell lines or the use of hypoxic conditions or chemical mimics for production from traditionally derived transduced producer lines.

(v) it enables the production of SIN vectors by stable cell line producer technology. Previously, SIN vectors have not been amenable to production by stable cell line producer technology because the deletion of the 3'U3 sequence resulted in at least a tenfold lower

titre of self-inactivating (SIN) vectors in comparison with vectors having intact LTRs. Consequently, SIN vectors have had to be prepared using transfection-based transient expression systems.

PRODUCER CELL

The high titre regulated retroviral vector particles of the present invention are typically generated in a suitable producer cell. Producer cells are generally mammalian cells but can be, for example, insect cells. A producer cell may be a packaging cell containing the virus structural genes, normally integrated into its genome into which the regulated retroviral vectors of the present invention are introduced. Alternatively the producer cell may be transfected with nucleic acid sequences encoding structural components, such as *gag/pol/env* on one or more vectors such as plasmids, adenovirus vectors, herpes viral vectors or any method known to deliver functional DNA into target cells. The vectors according to the present invention are then introduced into the packaging cell by the methods of the present invention.

As used herein, the term "producer cell" or "vector producing cell" refers to a cell which contains all the elements necessary for production of regulated retroviral vector particles and regulated retroviral delivery systems.

Preferably, the producer cell is obtainable from a stable producer cell line.

Preferably, the producer cell is obtainable from a derived stable producer cell line.

Preferably, the producer cell is obtainable from a derived producer cell line

As used herein, the term "derived producer cell line" is a transduced producer cell line which has been screened and selected for high expression of a marker gene. Such cell lines contain retroviral insertions in integration sites that support high level expression from the retroviral genome. The term "derived producer cell line" is used interchangeably with the term "derived stable producer cell line" and the term "stable producer cell line

Preferably the derived producer cell line includes but is not limited to a retroviral and/or a lentiviral producer cell.

Preferably the derived producer cell line is an HIV or EIAV producer cell line, more preferably an EIAV producer cell line.

Preferably the envelope protein sequences, and nucleocapsid sequences are all stably integrated in the producer and/or packaging cell. However, one or more of these sequences could also exist in episomal form and gene expression could occur from the episome.

PACKAGING CELL

As used herein, the term "packaging cell" refers to a cell which contains those elements necessary for production of infectious recombinant virus which are lacking in a recombinant viral vector. Typically, such packaging cells contain one or more expression cassettes which are capable of expressing viral structural proteins (such as *gag, pol* and *env*) but they do not contain a packaging signal.

The term "packaging signal" which is refered to interchangeably as "packaging sequence" or "*psi*" is used in reference to the non-coding sequence required for encapsidation of retroviral RNA strands during viral particle formation.

Packaging cell lines suitable for use with the above-described vector constructs may be readily prepared (see also WO 92/05266), and utilised to create producer cell lines for the production of retroviral vector particles. As already mentioned, a summary of the available packaging lines is presented in "Retroviruses" (1997 Cold Spring Harbour Laboratory Press Eds: JM Coffin, SM Hughes, HE Varmus pp 449).

The packaging cell lines are useful for providing the gene products necessary to encapsidate and provide a membrane protein for a high titre regulated retrovirus vector and regulated nucleic gene delivery vehicle production. When regulated retrovirus sequences are introduced into the packaging cell lines, such sequences are encapsidated

with the nucleocapsid (*gag/pol*) proteins and these units then bud through the cell membrane to become surrounded in cell membrane and to contain the envelope protein produced in the packaging cell line. These infectious regulated retroviruses are useful as infectious units *per se* or as gene delivery vectors.

The packaging cell may be a cell cultured *in vitro* such as a tissue culture cell line. Suitable cell lines include but are not limited to mammalian cells such as murine fibroblast derived cell lines or human cell lines. Preferably the packaging cell line is a human cell line, such as for example: HEK293, 293-T, TE671, HT1080.

Alternatively, the packaging cell may be a cell derived from the individual to be treated such as a monocyte, macrophage, blood cell or fibroblast. The cell may be isolated from an individual and the packaging and vector components administered *ex vivo* followed by re-administration of the autologous packaging cells.

Methods for introducing retroviral packaging and vector components into packaging/producer cells are described in the present invention.

Preferably the method of the present invention utilises a recombinase assisted mechanism.

Preferably the method of the present invention utilises a recombinase assisted mechanism which facilitates the production of high titre regulated retroviral vectors from the producer cells of the present invention.

RECOMBINASE ASSISTED MECHANISM

As used herein, the term "recombinase assisted system" includes but is not limited to a system using the Cre recombinase / loxP recognition sites of bacteriophage P1 or the site-specific FLP recombinase of *S. cerevisiae* which catalyses recombination events between 34 bp FLP recognition targets (FRTs).

The site-specific FLP recombinase of *S. cerevisiae* which catalyses recombination events between 34 bp FLP recognition targets (FRTs) has been configured into DNA constructs

in order to generate high level producer cell lines using recombinase-assisted recombination events (Karreman *et al.* (1996) NAR 24, 1616-1624). A similar system has been developed using the Cre recombinase / loxP recognition sites of bacteriophage P1. This was configured into a retroviral genome such that high titre retroviral producer cell lines were generated (Vanin *et al.* (1997) J Virol 71, 7820-7826). However, the use of the second method (Vanin *et al ibid*) has centered around the exchange of the central portions of a retroviral cassette using a recombinase-assisted system. Moreover, these methods have used genes encoding selectable markers such as neo^R and $puro^R$ (Vanin *et al ibid*) and luciferase and $puro^R$ linked by an IRES sequence (Karreman *et al ibid*). Karreman and Vanin do not demonstrate or *suggest* that: (i) a regulated or inactive 3'U3 sequence of the 3'LTR can be introduced into a producer cell via a recombinase-assisted mechanism or (ii) that therapeutic genes under the control of a regulated LTR may be introduced into a producer cell line via a recombinase assisted step. Vanin *et al ibid* suggests that his Cre-mediated recombination approach to retroviral producer cell line production may be used in combination with other modifications which should result in improved vector performance. Vanin *et al ibid* also suggests that his approach provides a means to generate high titre SIN vectors. However, there is no worked example and in fact no enabling disclosure because the skilled person would not have been aware, on the basis of the Vanin *et al* paper, of the necessary modifications to make the suggested approach work. Vanin *et al* makes no reference to hypoxic regulated vectors and/or regulated/inactivated lentiviral vectors.

LTRs

As already indicated, each retroviral genome comprises genes called *gag*, *pol* and *env* which code for virion proteins and enzymes. In the provirus, these genes are flanked at both ends by regions called long terminal repeats (LTRs). The LTRs are responsible for proviral integration, and transcription. They also serve as enhancer-promoter sequences. In other words, the LTRs can control the expression of the viral gene. Encapsidation of the retroviral RNAs occurs by virtue of a *psi* sequence located at the 5' end of the viral genome.

As used herein, the term "long terminal repeat (LTR) is used in reference to domains of base pairs located at the end of retroviral DNAs.

The LTRs themselves are identical sequences that can be divided into three elements, which are called U3, R and U5. U3 is derived from the sequence unique to the 3' end of the RNA. R is derived from a sequence repeated at both ends of the RNA and U5 is derived from the sequence unique to the 5' end of the RNA. The sizes of the three elements can vary considerably among different retroviruses.

For ease of understanding, a simple, generic structures (not to scale) of the RNA and the DNA forms of the MLV retroviral genome is presented in Figure 7 in which the elementary features of the LTRs and the relative positioning of *gag/pol* and *env* are indicated. Please note that (i) gag/pol and env are normally not spaced apart; and (ii) the overlap normally present between the *pol* and *env* genes and the poly A tail normally present at the 3' end of the RNA transcript are not illustrated in Figure 7.

As shown in Figure 7, the basic molecular organisation of an infectious retroviral RNA genome is (5') R - U5 - *gag/pol*, *env* - U3-R (3'). In a defective retroviral vector genome *gag*, *pol* and *env* may be absent or not functional. The R regions at both ends of the RNA are repeated sequences. U5 and U3 represent unique sequences at the 5' and 3' ends of the RNA genome respectively.

Upon cellular transduction, reverse transcription of the virion RNA into double stranded DNA takes place in the cytoplasm and involves two jumps of the reverse transcriptase from the 5' terminus to the 3' terminus of the template molecule. The result of these jumps is a duplication of sequences located at the 5' and 3' ends of the virion RNA. These sequences then occur fused in tandem on both ends of the viral DNA, forming the long terminal repeats (LTRs) which comprise R U5 and U3 regions. On completion of the reverse transcription, the viral DNA is translocated into the nucleus where the linear copy of the retroviral genome, called a preintegration complex (PIC), is randomly inserted into chromosomal DNA with the aid of the virion integrase to form a stable provirus. The number of possible sites of integration into the host cellular genome is very large and very widely distributed.

Preferably the retroviral genome is introduced into packaging cell lines using retroviral transduction.

Preferably retroviral vector particles (such as MLV vector particles) are prepared in a transient expression system with a different envelope pseudotype to the packaging cell, and used to transduce a retroviral packaging cell.

Preferably the retroviral transduction step identifies retroviral insertions in integration sites that support high level expression of the resulting regulated retroviral genome.

Preferably stable transduced producer cell lines made by this initial retroviral transduction step produce retrovirus at titres of at least 10^6 per ml, such as from about 10^6 to about 10^7 per ml, more preferably at least about 10^7 per ml.

HIGH TITRE

As used herein, the term "high titre" means an effective amount of a retroviral vector or particle which is capable of transducing a target site such as a cell.

As used herein, the term "effective amount" means an amount of a regulated retroviral or lentiviral vector or vector particle which is sufficient to induce expression of an NOI at a target site.

Preferably the titre is from at least 10^6 retrovirus particles per ml, such as from about 10^6 to about 10^7 per ml, more preferably at least about 10^7 retrovirus particles per ml.

TRANSCRIPTIONAL CONTROL

The control of proviral transcription remains largely with the noncoding sequences of the viral LTR. The site of transcription initiation is at the boundary between U3 and R in the left hand side LTR (as shown in Figure 7) and the site of poly (A) addition (termination) is at the boundary between R and U5 in the right hand side LTR (as shown in Figure 7). The 3'U3 sequence contains most of the transcriptional control elements of the provirus,

which include the promoter and multiple enhancer sequences responsive to cellular and in some cases, viral transcriptional activator proteins.

REGULTABLE LTRs

AN LTR present, for example, in the construct of the invention and as a 3'LTR in the provirus of the producer cell of the invention may be a native LTR or a heterologous regulatable LTR. It may also be a transcriptionally quiescent LTR for use in SIN vector technology.

As used herein, the terms "regulatable LTR" and "regulatable 3'LTR" include vectors which contain responsive elements which are present in retroviral 3' LTR sequences, either by design or by their nature. As used herein, vectors comprising a "regulatable 3'LTR" are referred to as "regulated retroviral vectors". Within the regulatable 3'LTR region, the 3'U3 sequence contains most of the transcriptional control elements of the provirus, which include the promoter and multiple enhancer sequences responsive to cellular and in some cases, viral transcriptional activator proteins.

Responsive elements include but are not limited to elements which comprise, for example, promoter and multiple enhancer sequences responsive to cellular and in some cases, viral transcriptional activator proteins and/or elements which have been modified to render them inactive. As used herein, the term "modified" includes but is not limited to silencing, disabling, mutating, deleting or removing all of the U3 sequence or a part thereof.

The term "regulated LTR" also includes an inactive LTR such that the resulting provirus in the target cell can not produce a packagable viral genome (self-inactivating (SIN) vector technology) - see the Examples and Figure 6 for a particular embodiment.

ENHANCER

As used herein, the term "enhancer" includes a DNA sequence which binds other protein components of the transcription initiation complex and thus facilitates the initiation of transcription directed by its associated promoter.

In one preferred embodiment of the present invention, the enhancer is an ischaemic like response element (ILRE).

ILRE

The term "ischaemia like response element" - otherwise written as ILRE - includes an element that is responsive to or is active under conditions of ischaemia or conditions that are like ischaemia or are caused by ischaemia. By way of example, conditions that are like ischaemia or are caused by ischaemia include hypoxia and/or low glucose concentration(s).

The term "hypoxia" means a condition under which a particular organ or tissue receives an inadequate supply of oxygen.

Ischaemia can be an insufficient supply of blood to a specific organ or tissue. A consequence of decreased blood supply is an inadequate supply of oxygen to the organ or tissue (hypoxia). Prolonged hypoxia may result in injury to the affected organ or tissue.

A preferred ILRE is an hypoxia response element (HRE).

HRE

In one preferred aspect of the present invention, there is hypoxia or ischaemia regulatable expression of the retroviral vector components. In this regard, hypoxia is a powerful regulator of gene expression in a wide range of different cell types and acts by the induction of the activity of hypoxia-inducible transcription factors such as hypoxia inducible factor-1 (HIF-1; Wang & Semenza 1993 Proc Natl Acad Sci 90:430), which

bind to cognate DNA recognition sites, the hypoxia-responsive elements (HREs) on various gene promoters. Dachs *et al* (1997 Nature Med 5: 515) have used a multimeric form of the HRE from the mouse phosphoglycerate kinase-1 (PGK-1) gene (Firth *et al* 1994 Proc Natl Acad Sci 91:6496-6500) to control expression of both marker and therapeutic genes by human fibrosarcoma cells in response to hypoxia *in vitro* and within solid tumours *in vivo* (Dachs *et al ibid*).

Hypoxia response enhancer elements (HREEs) have also been found in association with a number of genes including the erythropoietin (EPO) gene (Madan *et al* 1993 Proc Natl Acad Sci 90: 3928; Semenza and Wang 1992 Mol Cell Biol 1992 12: 5447-5454). Other HREEs have been isolated from regulatory regions of both the muscle glycolytic enzyme pyrivate kinase (PKM) gene (Takenaka *et al* 1989 J Biol Chem 264: 2363-2367), the human muscle-specific β-enolase gene (ENO3; Peshavaria and Day 1991 Biochem J 275: 427-433) and the endothelin-1 (ET-1) gene (Inoue *et al* 1989 J Biol Chem 264: 14954-14959).

Preferably the HRE of the present invention is selected from, for example, the erythropoietin HRE element (HREE1), muscle pyruvate kinase (PKM), HRE element, phosphoglycerate kinase (PGK) HRE, B-enolase (enolase 3; ENO3) HRE element, endothelin-1 (ET-1)HRE element and metallothionein II (MTII) HRE element.

RESPONSIVE ELEMENT

Preferably the ILRE is used in combination with a transcriptional regulatory element , such as a promoter, which transcriptional regulatory element is preferably active in one or more selected cell type(s), preferably being only active in one cell type.

As outlined above, this combination aspect of the present invention is called a responsive element.

Preferably the responsive element comprises at least the ILRE as herein defined.

Non-limiting examples of such a responsive element are presented as OBHRE1 and XiaMac. Another non-limiting example includes the ILRE in use in conjunction with an MLV promoter and/or a tissue restricted ischaemic responsive promoter. These responsive elements are disclosed in WO99/15684.

Other examples of suitable tissue restricted promoters/enhancers are those which are highly active in tumour cells such as a promoter/enhancer from a *MUC1* gene, a *CEA* gene or a *5T4* antigen gene. The alpha fetoprotein (AFP) promoter is also a tumour-specific promoter. One preferred promoter-enhancer combination is a human cytomegalovirus (hCMV) major immediate early (MIE) promoter/enhancer combination.

PROMOTER

The term "promoter" is used in the normal sense of the art, e.g. an RNA polymerase binding site.

The promoter may be located in the retroviral 5' LTR to control the expresssion of a cDNA encoding an NOI.

Preferably the NOI is capable of being expressed from the retrovirus genome such as from endogenous retroviral promoters in the long terminal repeat (LTR)

Preferably the NOI is expressed from a heterologous promoter to which the heterologous gene or sequence is operably linked.

Alternatively, the promoter may be an internal promoter.

Preferably the NOI is expressed from an internal promoter.

Vectors containing internal promoters have also been widely used to express multiple genes. An internal promoter makes it possible to exploit promoter/enhancer combinations other than those found in the viral LTR for driving gene expression. Multiple internal promoters can be included in a retroviral vector and it has proved

possible to express at least three different cDNAs each from its own promoter (Overell *et al* 1988 Mol Cell Biol 8: 1803-1808). Internal ribosomal entry site (IRES) elements have also been used to allow translation of multiple coding regions from either a single mRNA or from fusion proteins that can then be expressed from an open reading frame.

TISSUE SPECIFIC PROMOTERS

The promoter of the present invention may be constitutively efficient, or may be tissue or temporally restricted in their activity.

Preferably the promoter is a constitutive promoter such as CMV.

Preferably the promoters of the present invention are tissue specific.

That is, they are capable of driving transcription of a NOI or NOI(s) in one tissue while remaining largely "silent" in other tissue types.

The term "tissue specific" means a promoter which is not restricted in activity to a single tissue type but which nevertheless shows selectivity in that they may be active in one group of tissues and less active or silent in another group.

The level of expression of an NOI or NOIs under the control of a particular promoter may be modulated by manipulating the promoter region. For example, different domains within a promoter region may possess different gene regulatory activities. The roles of these different regions are typically assessed using vector constructs having different variants of the promoter with specific regions deleted (that is, deletion analysis). This approach may be used to identify, for example, the smallest region capable of conferring tissue specificity or the smallest region conferring hypoxia sensitivity.

A number of tissue specific promoters, described above, may be particularly advantageous in practising the present invention. In most instances, these promoters may be isolated as convenient restriction digestion fragments suitable for cloning in a selected vector. Alternatively, promoter fragments may be isolated using the polymerase chain

reaction. Cloning of the amplified fragments may be facilitated by incorporating restriction sites at the 5' end of the primers.

The NOI or NOIs may be under the expression control of an expression regulatory element, such as a promoter and enhancer.

Preferably the ischaemic responsive promoter is a tissue restricted ischaemic responsive promoter.

Preferably the tissue restricted ischaemic responsive promoter is a macrophage specific promoter restricted by repression.

Preferably the tissue restricted ischaemic responsive promoter is an endothelium specific promoter.

Preferably the regulated retroviral vector of the present invention is an ILRE regulated retroviral vector.

Preferably the regulated retroviral vector of the present invention is an ILRE regulated lentiviral vector.

Preferably the regulated retroviral vector of the present invention is an autoregulated hypoxia responsive lentiviral vector.

Preferably the regulated retroviral vector of the present invention is regulated by glucose concentration.

For example, the glucose-regulated proteins (grp's) such as grp78 and grp94 are highly conserved proteins known to be induced by glucose deprivation (Attenello and Lee 1984 Science 226 187-190). The grp 78 gene is expressed at low levels in most normal healthy tissues under the influence of basal level promoter elements but has at least two critical "stress inducible regulatory elements" upstream of the TATA element (Attenello 1984 ibid; Gazit *et al* 1995 Cancer Res 55: 1660-1663). Attachment to a truncated 632 base

pair sequence of the 5'end of the grp78 promoter confers high inducibility to glucose deprivation on reporter genes *in vitro* (Gazit *et al* 1995 *ibid*). Furthermore, this promoter sequence in retroviral vectors was capable of driving a high level expression of a reporter gene in tumour cells in murine fibrosarcomas, particularly in central relatively ischaemic/fibrotic sites (Gazit *et al* 1995 *ibid*).

Preferably the regulated retroviral vector of the present invention is a self-inactivating (SIN) vector.

By way of example, self-inactivating retroviral vectors have been constructed by deleting the transcriptional enhancers or the enhancers and promoter in the U3 region of the 3' LTR. After a round of vector reverse transcription and integration, these changes are copied into both the 5' and the 3' LTRs producing a transcriptionally inactive provirus (Yu *et al* 1986 Proc Natl Acad Sci 83: 3194-3198; Dougherty and Temin 1987 Proc Natl Acad Sci 84: 1197-1201; Hawley *et al* 1987 Proc Natl Acad Sci 84: 2406-2410; Yee *et al* 1987 Proc Natl Acad Sci 91: 9564-9568). However, any promoter(s) internal to the LTRs in such vectors will still be transcriptionally active. This strategy has been employed to eliminate effects of the enhancers and promoters in the viral LTRs on transcription from internally placed genes. Such effects include increased transcription (Jolly *et al* 1983 Nucleic Acids Res 11: 1855-1872) or suppression of transcription (Emerman and Temin 1984 Cell 39: 449-467). This strategy can also be used to eliminate downstream transcription from the 3' LTR into genomic DNA (Herman and Coffin 1987 Science 236: 845-848). This is of particular concern in human gene therapy where it is of critical importance to prevent the adventitious activation of an endogenous oncogene.

RETROVIRAL VECTORS

The regulated retroviral vector of the present invention includes but is not limited to: murine leukemia virus (MLV), human immunodeficiency virus (HIV), equine infectious anaemia virus (EIAV), feline immunodeficiency virus (FIV), caprine encephalitis-arthritis virus (CAEV), mouse mammary tumour virus (MMTV), Rous sarcoma virus (RSV), Fujinami sarcoma virus (FuSV), Moloney murine leukemia virus (Mo-MLV), FBR murine osteosarcoma virus (FBR MSV), Moloney murine sarcoma virus (Mo-MSV),

Abelson murine leukemia virus (A-MLV), Avian myelocytomatosis virus-29 (MC29), and Avian erythroblastosis virus (AEV).

A detailed list of retroviruses may be found in Coffin *et al* ("Retroviruses" 1997 Cold Spring Harbour Laboratory Press Eds: JM Coffin, SM Hughes, HE Varmus pp 758-763).

Preferred vectors for use in accordance with the present invention are retroviral vectors, such as MLV vectors.

Preferably the recombinant retroviral vectors of the present invention are lentiviral vectors, more preferably HIV or EIAV vectors.

LENTIVIRAL VECTORS

The lentiviruses can be divided into primate and non-primate groups. Examples of primate lentiviruses include but are not limited to: the human immunodeficiency virus (HIV), the causative agent of human auto-immunodeficiency syndrome (AIDS), and the simian immunodeficiency virus (SIV). The non-primate lentiviral group includes the prototype "slow virus" visna/maedi virus (VMV), as well as the related caprine arthritis-encephalitis virus (CAEV), equine infectious anaemia virus (EIAV) and the more recently described feline immunodeficiency virus (FIV) and bovine immunodeficiency virus (BIV).

A distinction between the lentivirus family and other types of retroviruses is that lentiviruses have the capability to infect both dividing and non-dividing cells (Lewis *et al* 1992 EMBO. J 11: 3053-3058; Lewis and Emerman 1994 J. Virol. 68: 510-516). In contrast, other retroviruses - such as MLV - are unable to infect non-dividing cells such as those that make up, for example, muscle, brain, lung and liver tissue.

Preferred vectors for use in accordance with the present invention are recombinant retroviral vectors, in particular recombinant lentiviral vectors, in particular minimal lentiviral vectors which are disclosed in WO 99/32646 and in WO98/17815.

VECTOR

As used herein, a "vector" denotes a tool that allows or faciliates the transfer of an entity from one environment to another. In accordance with the present invention, and by way of example, some vectors used in recombinant DNA techniques allow entities, such as a segment of DNA (such as a heterologous DNA segment, such as a heterologous cDNA segment), to be transferred into a target cell. Optionally, once within the target cell, the vector may then serve to maintain the heterologous DNA within the cell or may act as a unit of DNA replication. Examples of vectors used in recombinant DNA techniques include plasmids, chromosomes, artificial chromosomes or viruses.

OPERABLY LINKED

The term "operably linked" denotes a relationship between a regulatory region (typically a promoter element, but may include an enhancer element) and the coding region of a gene, whereby the transcription of the coding region is under the control of the regulatory region.

DERIVABLE

The term "derivable" is used in its normal sense as meaning a nucleotide sequence such as an LTR or a part thereof which need not necessarily be obtained from an vector such as a retroviral vector but instead could be derived therefrom. By way of example, the sequence may be prepared synthetically or by use of recombinant DNA techniques.

VECTOR PARTICLES

In the present invention, several terms are used interchangeably. Thus, "virion", "virus", "viral particle", "retroviral particle", "retrovirus", and "vector particle" mean virus and virus-like particles that are capable of introducing a nucleic acid into a cell through a viral-like entry mechanism. Such vector particles can, under certain circumstances, mediate the transfer of NOIs into the cells they infect. A retrovirus is capable of reverse

transcribing its genetic material into DNA and incorporating this genetic material into a target cell's DNA upon transduction. Such cells are designated herein as "target cells".

A vector particle includes the following components: a retrovirus nucleic acid, which may contain one or more NOIs, a nucleocapsid encapsidating the nucleic acid, the nucleocapsid comprising nucleocapsid protein of a retrovirus, and a membrane surrounding the nucleocapsid.

NUCLEOCAPSID

The term "nucleocapsid" refers to at least the group specific viral core proteins (*gag*) and the viral polymerase (*pol*) of a retrovirus genome. These proteins encapsidate the retrovirus-packagable sequences and themselves are further surrounded by a membrane containing an envelope glycoprotein.

Preferably a high titre retroviral vector is produced using a codon optimised gag and a codon optimised pol or a codon optimised env.

CODON OPTIMISATION

As used herein, the terms "codon optimised" and "codon optimisation" refer to an improvement in codon usage. By way of example, alterations to the coding sequences for viral components may improve the sequences for codon usage in the mammalian cells or other cells which are to act as the producer cells for retroviral vector particle production. This is referred to as "codon optimisation". Many retroviruses, including HIV and other lentiviruses, use a large number of rare codons and by changing these to correspond to commonly used mammalian codons, increased expression of the packaging components in mammalian producer cells can be achieved. Codon usage tables are known in the art for mammalian cells, as well as for a variety of other organisms.

Preferably a high titre lentiviral vector is produced using a codon optimised gag and a codon optimised pol or a codon optimised env.

Preferably a high titre retroviral vector is produced using a modified and/or extended packaging signal.

PACKAGING SIGNAL

As used herein, the term "packaging signal" or "packaging sequence" refers to sequences located within the retroviral genome which are required for insertion of the viral RNA into the viral capsid or particle. Several retroviral vectors use the minimal packaging signal (also referred to as the psi sequence) needed for encapsidation of the viral genome. By way of example, this minimal packaging signal encompasses bases 212 to 563 of the Mo-MLV genome (Mann et al 1983: Cell 33: 153).

As used herein, the term "extended packaging signal" or "extended packaging sequence" refers to the use of sequences around the psi sequence with further extension into the gag gene. The inclusion of these additional packaging sequences may increase the efficiency of insertion of vector RNA into viral particles.

Preferably a high titre lentiviral vector is produced using a modified packaging signal.

Preferably the lentiviral construct is a based on an EIAV vector genome where all the accessory genes are removed except Rev.

ACCESSORY GENES

As used herein, the term "accessory genes" refer to a variety of virally encoded accessory proteins capable of modulating various aspects of retroviral replication and infectivity. These proteins are discussed in Coffin et al (ibid) (Chapters 6 and 7). Examples of accessory proteins in lentiviral vectors include but are not limited to tat, rev, nef, vpr, vpu, vif, vpx. An example of a lentiviral vector useful in the present invention is one which has all of the accessory genes removed except rev.

Preferably the production of lentiviral vector particles is increased by about 10 fold in the presence of EIAV Rev.

ENV

If the retroviral component includes an *env* nucleotide sequence, then all or part of that sequence can be optionally replaced with all or part of another *env* nucleotide sequence such as, by way of example, the amphotropic Env protein designated 4070A or the influenza haemagglutinin (HA) or the vesicular stomatitis virus G (VSV-G) protein. Replacement of the *env* gene with a heterologous *env* gene is an example of a technique or strategy called pseudotyping. Pseudotyping is not a new phenomenon and examples may be found in WO-A-98/05759, WO-A-98/05754, WO-A-97/17457, WO-A-96/09400, WO-A-91/00047 and Mebatsion *et al* 1997 Cell 90, 841-847.

In one preferred aspect, the retroviral vector of the present invention has been pseudotyped. In this regard, pseudotyping can confer one or more advantages. For example, with the lentiviral vectors, the *env* gene product of the HIV based vectors would restrict these vectors to infecting only cells that express a protein called CD4. But if the *env* gene in these vectors has been substituted with *env* sequences from other RNA viruses, then they may have a broader infectious spectrum (Verma and Somia 1997 Nature 389:239-242). By way of example, workers have pseudotyped an HIV based vector with the glycoprotein from VSV (Verma and Somia 1997 *ibid*).

In another alternative, the Env protein may be a modified Env protein such as a mutant or engineered Env protein. Modifications may be made or selected to introduce targeting ability or to reduce toxicity or for another purpose (Valsesia-Wittman *et al* 1996 J Virol 70: 2056-64; Nilson *et al* 1996 Gene Therapy 3: 280-6; Fielding *et al* 1998 Blood 9: 1802 and references cited therein).

TARGET CELL

As used herein the term "target cell" simply refers to a cell which the regulated retroviral vector of the present invention, whether native or targeted, is capable of infecting or transducing.

The lentiviral vector particle according to the invention will be capable of transducing cells which are slowly-dividing, and which non-lentiviruses such as MLV would not be able to efficiently transduce. Slowly-dividing cells divide once in about every three to four days including certain tumour cells. Although tumours contain rapidly dividing cells, some tumour cells especially those in the centre of the tumour, divide infrequently.

Alternatively the target cell may be a growth-arrested cell capable of undergoing cell division such as a cell in a central portion of a tumour mass or a stem cell such as a haematopoietic stem cell or a CD34-positive cell.

As a further alternative, the target cell may be a precursor of a differentiated cell such as a monocyte precursor, a CD33-positive cell, or a myeloid precursor.

As a further alternative, the target cell may be a differentiated cell such as a neuron, astrocyte, glial cell, microglial cell, macrophage, monocyte, epithelial cell, endothelial cell, hepatocyte, spermatocyte, spermatid or spermatozoa.
Target cells may be transduced either *in vitro* after isolation from a human individual or may be transduced directly *in vivo*.

NOI

In accordance with the present invention, it is possible to manipulate the viral genome or the regulated retroviral vector nucleotide sequence, so that viral genes are replaced or supplemented with one or more NOIs which may be heterologous NOIs.

The term "heterologous" refers to a nucleic acid sequence or protein sequence linked to a nucleic acid or protein sequence which it is not naturally linked.

With the present invention, the term NOI (i.e. nucleotide sequence of interest) includes any suitable nucleotide sequence, which need not necessarily be a complete naturally occurring DNA sequence. Thus, the DNA sequence can be, for example, a synthetic DNA sequence, a recombinant DNA sequence (i.e. prepared by use of recombinant DNA techniques), a cDNA sequence or a partial genomic DNA sequence, including

combinations thereof. The DNA sequence need not be a coding region. If it is a coding region, it need not be an entire coding region. In addition, the DNA sequence can be in a sense orientation or in an anti-sense orientation. Preferably, it is in a sense orientation. Preferably, the DNA is or comprises cDNA.

The NOI(s) may be any one or more of selection gene(s), marker gene(s) and therapeutic gene(s). As used herein, the term "selection gene" refers to the use of a NOI which encodes a selectable marker which may have an enzymatic activity that confers resistance to an antibiotic or drug upon the cell in which the selectable marker is expressed.

SELECTABLE MARKERS

Many different selectable markers have been used successfully in retroviral vectors. These are reviewed in "Retroviruses" (1997 Cold Spring Harbour Laboratory Press Eds: JM Coffin, SM Hughes, HE Varmus pp 444) and include, but are not limited to, the bacterial neomycin (neo) and hygromycin phosphotransferase genes which confer resistance to G418 and hygromycin respectively; a mutant mouse dihydrofolate reductase gene which confers resistance to methotrexate; the bacterial *gpt* gene which allows cells to grow in medium containing mycophenolic acid, xanthine and aminopterin; the bacterial *hisD* gene which allows cells to grow in medium without histidine but containing histidinol; the multidrug resistance gene (*mdr*) which confers resistance to a variety of drugs; and the bacterial genes which confer resistance to puromycin or phleomycin. All of these markers are dominant selectable and allow chemical selection of most cells expressing these genes. Other selectable markers are not dominant in that their use must be in conjunction with a cell line that lacks the relevant enzyme activity. Examples of non-dominant selectable markers include the thymidine kinase (*tk*) gene which is used in conjunction with *tk* cell lines.

Particularly preferred markers are blasticidin and neomycin, optionally operably linked to a thymidine kinase coding sequence typically under the transcriptional control of a strong viral promoter such the SV40 promoter.

NOIs WITH THERAPEUTIC AND/OR DIAGNOSTIC APPLICATIONS

In accordance with the present invention, suitable NOI sequences include those that are of therapeutic and/or diagnostic application such as, but are not limited to: sequences encoding cytokines, chemokines, hormones, antibodies, engineered immunoglobulin-like molecules, a single chain antibody, fusion proteins, enzymes, immune co-stimulatory molecules, immunomodulatory molecules, anti-sense RNA, a transdominant negative mutant of a target protein, a toxin, a conditional toxin, an antigen, a tumour suppressor protein and growth factors, membrane proteins, vasoactive proteins and peptides, anti-viral proteins and ribozymes, and derivatives therof (such as with an associated reporter group). When included, such coding sequences may be typically operatively linked to a suitable promoter. which may be a promoter driving expression of a ribozyme(s), or a different promoter or promoters, such as in one or more specific cell types.

NOIs FOR TREATING CANCER

Suitable NOIs for use in the invention in the treatment or prophylaxis of cancer include NOIs encoding proteins which: destroy the target cell (for example a ribosomal toxin), act as: tumour suppressors (such as wild-type p53); activators of anti-tumour immune mechanisms (such as cytokines, co-stimulatory molecules and immunoglobulins); inhibitors of angiogenesis; or which provide enhanced drug sensitivity (such as pro-drug activation enzymes); indirectly stimulate destruction of target cell by natural effector cells (for example, strong antigen to stimulate the immune system or convert a precursor substance to a toxic substance which destroys the target cell (for example a prodrug activating enzyme).

PRO-DRUG ACTIVATING ENZYMES

Examples of prodrugs include but are not limited to etoposide phosphate (used with alkaline phosphatase; 5-fluorocytosine (with cytosine deaminase); Doxorubin-N-p-hydroxyphenoxyacetamide (with Penicillin-V-Amidase); Para-N-bis (2-chloroethyl)aminobenzoyl glutamate (with Carboxypeptidase G2); Cephalosporin nitrogen mustard carbamates (with B-lactamase); SR4233 (with p450 reductase);

Ganciclovir (with HSV thymidine kinase); mustard pro-drugs with nitroreductase and cyclophosphamide or ifosfamide (with cytochrome p450).

NOIs FOR TREATING HEART DISEASE

Suitable NOIs for use in the treatment or prevention of ischaemic heart disease include NOIs encoding plasminogen activators. Suitable NOIs for the treatment or prevention of rheumatoid arthritis or cerebral malaria include genes encoding anti-inflammatory proteins, antibodies directed against tumour necrosis factor (TNF) alpha, and anti-adhesion molecules (such as antibody molecules or receptors specific for adhesion molecules).

BYSTANDER EFFECT

The expression products encoded by the NOIs may be proteins which are secreted from the cell. Alternatively the NOI expression products are not secreted and are active within the cell. In either event, it is preferred for the NOI expression product to demonstrate a bystander effector or a distant bystander effect; that is the production of the expression product in one cell leading to the killing of additional, related cells, either neighbouring or distant (e.g. metastatic), which possess a common phenotype. Encoded proteins could also destroy bystander tumour cells (for example with secreted antitumour antibody-ribosomal toxin fusion protein), indirectly stimulated destruction of bystander tumour cells (for example cytokines to stimulate the immune system or procoagulant proteins causing local vascular occlusion) or convert a precursor substance to a toxic substance which destroys bystander tumour cells (eg an enzyme which activates a prodrug to a diffusible drug). Also, the delivery of NOI(s) encoding antisense transcripts or ribozymes which interfere with expression of cellular genes for tumour persistence (for example against aberrant *myc* transcripts in Burkitts lymphoma or against *bcr-abl* transcripts in chronic myeloid leukemia. The use of combinations of such NOIs is also envisaged.

CYTOKINES

The NOI or NOIs of the present invention may also comprise one or more cytokine-encoding NOIs. Suitable cytokines and growth factors include but are not limited to: ApoE, Apo-SAA, BDNF, Cardiotrophin-1, EGF, ENA-78, Eotaxin, Eotaxin-2, Exodus-2, FGF-acidic, FGF-basic, fibroblast growth factor-10 (Marshall 1998 Nature Biotechnology 16: 129).FLT3 ligand (Kimura *et al* (1997), Fractalkine (CX3C), GDNF, G-CSF, GM-CSF, GF-β1, insulin, IFN-γ, IGF-I, IGF-II, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8 (72 a.a.), IL-8 (77 a.a.), IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, IL-16, IL-17, IL-18 (IGIF), Inhibin α, Inhibin β, IP-10, keratinocyte growth factor-2 (KGF-2), KGF, Leptin, LIF, Lymphotactin, Mullerian inhibitory substance, monocyte colony inhibitory factor, monocyte attractant protein (Marshall 1998 *ibid*), M-CSF, MDC (67 a.a.), MDC (69 a.a.), MCP-1 (MCAF), MCP-2, MCP-3, MCP-4, MDC (67 a.a.), MDC (69 a.a.), MIG, MIP-1α, MIP-1β, MIP-3α, MIP-3β, MIP-4, myeloid progenitor inhibitor factor-1 (MPIF-1), NAP-2, Neurturin, Nerve growth factor, β-NGF, NT-3, NT-4, Oncostatin M, PDGF-AA, PDGF-AB, PDGF-BB, PF-4, RANTES, SDF1α, SDF1β, SCF, SCGF, stem cell factor (SCF), TARC, TGF-α, TGF-β, TGF-β2, TGF-β3, tumour necrosis factor (TNF), TNF-α, TNF-β, TNIL-1, TPO, VEGF, GCP-2, GRO/MGSA, GRO-β, GRO-γ, HCC1, 1-309.

The NOI or NOIs may be under the expression control of an expression regulatory element, such as a promoter and/or a promoter enhancer as known as "responsive elements" in the present invention.

VIRAL DELIVERY SYSTEMS

When the regulated retroviral vector particles are used to transfer NOIs into cells which they transduce, such vector particles also designated "viral delivery systems" or "retroviral delivery systems". Viral vectors, including retroviral vectors, have been used to transfer NOIs efficiently by exploiting the viral transduction process. NOIs cloned into the retroviral genome can be delivered efficiently to cells susceptible to transduction by a retrovirus. Through other genetic manipulations, the replicative capacity of the retroviral

genome can be destroyed. The vectors introduce new genetic material into a cell but are unable to replicate.

The regulated retroviral vector of the present invention can be delivered by viral or non-viral techniques. Non-viral delivery systems include but are not limted to DNA transfection methods. Here, transfection includes a process using a non-viral vector to deliver a gene to a target mammalian cell.

Typical transfection methods include electroporation, DNA biolistics, lipid-mediated transfection, compacted DNA-mediated transfection, liposomes, immunoliposomes, lipofectin, cationic agent-mediated, cationic facial amphiphiles (CFAs) (Nature Biotechnology 1996 14; 556), multivalent cations such as spermine, cationic lipids or polylysine, 1, 2,-bis (oleoyloxy)-3-(trimethylammonio) propane (DOTAP)-cholesterol complexes (Wolff and Trubetskoy 1998 Nature Biotechnology 16: 421) and combinations thereof.

Viral delivery systems include but are not limited to adenovirus vector, an adeno-associated viral (AAV) vector, a herpes viral vector, a retroviral vector, a lentiviral vector, or a baculoviral vector. These viral delivery systems may be configured as a split-intron vector. A split intron vector is described in WO 99/15683.

Other examples of vectors include *ex vivo* delivery systems, which include but are not limited to DNA transfection methods such as electroporation, DNA biolistics, lipid-mediated transfection, compacted DNA-mediated transfection.

The vector may be a plasmid DNA vector. Alternatively, the vector may be a recombinant viral vector. Suitable recombinant viral vectors include adenovirus vectors, adeno-associated viral (AAV) vectors, Herpes-virus vectors, or retroviral vectors, lentiviral vectors or a combination of adenoviral and lentiviral vectors. In the case of viral vectors, gene delivery is mediated by viral infection of a target cell.

If the features of adenoviruses are combined with the genetic stability of retro/lentiviruses then essentially the adenovirus can be used to transduce target cells to become transient retroviral producer cells that could stably infect neighbouring cells.

PHARMACEUTICAL COMPOSITION

The present invention also provides a pharmaceutical composition for treating an individual by gene therapy, wherein the composition comprises a therapeutically effective amount of a regulated retroviral vector according to the present invention. The pharmaceutical composition may be for human or animal usage. Typically, a physician will determine the actual dosage which will be most suitable for an individual subject and it will vary with the age, weight and response of the particular patient.

The composition may optionally comprise a pharmaceutically acceptable carrier, diluent, excipient or adjuvant. The choice of pharmaceutical carrier, excipient or diluent can be selected with regard to the intended route of administration and standard pharmaceutical practice. The pharmaceutical compositions may comprise as - or in addition to - the carrier, excipient or diluent any suitable binder(s), lubricant(s), suspending agent(s), coating agent(s), solubilising agent(s), and other carrier agents that may aid or increase the viral entry into the target site (such as for example a lipid delivery system).

Where appropriate, the pharmaceutical compositions can be administered by any one or more of: minipumps, inhalation, in the form of a suppository or pessary, topically in the form of a lotion, solution, cream, ointment or dusting powder, by use of a skin patch, orally in the form of tablets containing excipients such as starch or lactose, or in capsules or ovules either alone or in admixture with excipients, or in the form of elixirs, solutions or suspensions containing flavouring or colouring agents, or they can be injected parenterally, for example intracavernosally, intravenously, intramuscularly or subcutaneously. For parenteral administration, the compositions may be best used in the form of a sterile aqueous solution which may contain other substances, for example enough salts or monosaccharides to make the solution isotonic with blood. For buccal or sublingual administration the compositions may be administered in the form of tablets or lozenges which can be formulated in a conventional manner.

DISORDERS

The present invention is believed to have a wide therapeutic applicability - depending on *inter alia* the selection of the one or more NOIs.

For example, the present invention may be useful in the treatment of the disorders listed in WO-A-98/05635. For ease of reference, part of that list is now provided: cancer, inflammation or inflammatory disease, dermatological disorders, fever, cardiovascular effects, haemorrhage, coagulation and acute phase response, cachexia, anorexia, acute infection, HIV infection, shock states, graft-versus-host reactions, autoimmune disease, reperfusion injury, meningitis, migraine and aspirin-dependent anti-thrombosis; tumour growth, invasion and spread, angiogenesis, metastases, malignant, ascites and malignant pleural effusion; cerebral ischaemia, ischaemic heart disease, osteoarthritis, rheumatoid arthritis, osteoporosis, asthma, multiple sclerosis, neurodegeneration, Alzheimer's disease, atherosclerosis, stroke, vasculitis, Crohn's disease and ulcerative colitis; periodontitis, gingivitis; psoriasis, atopic dermatitis, chronic ulcers, epidermolysis bullosa; corneal ulceration, retinopathy and surgical wound healing; rhinitis, allergic conjunctivitis, eczema, anaphylaxis; restenosis, congestive heart failure, endometriosis, atherosclerosis or endosclerosis.

In addition, or in the alternative, the present invention may be useful in the treatment of disorders listed in WO-A-98/07859. For ease of reference, part of that list is now provided: cytokine and cell proliferation/differentiation activity; immunosuppressant or immunostimulant activity (e.g. for treating immune deficiency, including infection with human immune deficiency virus; regulation of lymphocyte growth; treating cancer and many autoimmune diseases, and to prevent transplant rejection or induce tumour immunity); regulation of haematopoiesis, e.g. treatment of myeloid or lymphoid diseases; promoting growth of bone, cartilage, tendon, ligament and nerve tissue, e.g. for healing wounds, treatment of burns, ulcers and periodontal disease and neurodegeneration; inhibition or activation of follicle-stimulating hormone (modulation of fertility); chemotactic/chemokinetic activity (e.g. for mobilising specific cell types to sites of injury or infection); haemostatic and thrombolytic activity (e.g. for treating haemophilia and stroke); antiinflammatory activity (for treating e.g. septic shock or Crohn's disease); as

antimicrobials; modulators of e.g. metabolism or behaviour; as analgesics; treating specific deficiency disorders; in treatment of e.g. psoriasis, in human or veterinary medicine.

In addition, or in the alternative, the present invention may be useful in the treatment of disorders listed in WO-A-98/09985. For ease of reference, part of that list is now provided: macrophage inhibitory and/or T cell inhibitory activity and thus, anti-inflammatory activity; anti-immune activity, i.e. inhibitory effects against a cellular and/or humoral immune response, including a response not associated with inflammation; inhibit the ability of macrophages and T cells to adhere to extracellular matrix components and fibronectin, as well as up-regulated fas receptor expression in T cells; inhibit unwanted immune reaction and inflammation including arthritis, including rheumatoid arthritis, inflammation associated with hypersensitivity, allergic reactions, asthma, systemic lupus erythematosus, collagen diseases and other autoimmune diseases, inflammation associated with atherosclerosis, arteriosclerosis, atherosclerotic heart disease, reperfusion injury, cardiac arrest, myocardial infarction, vascular inflammatory disorders, respiratory distress syndrome or other cardiopulmonary diseases, inflammation associated with peptic ulcer, ulcerative colitis and other diseases of the gastrointestinal tract, hepatic fibrosis, liver cirrhosis or other hepatic diseases, thyroiditis or other glandular diseases, glomerulonephritis or other renal and urologic diseases, otitis or other oto-rhino-laryngological diseases, dermatitis or other dermal diseases, periodontal diseases or other dental diseases, orchitis or epididimo-orchitis, infertility, orchidal trauma or other immune-related testicular diseases, placental dysfunction, placental insufficiency, habitual abortion, eclampsia, pre-eclampsia and other immune and/or inflammatory-related gynaecological diseases, posterior uveitis, intermediate uveitis, anterior uveitis, conjunctivitis, chorioretinitis, uveoretinitis, optic neuritis, intraocular inflammation, e.g. retinitis or cystoid macular oedema, sympathetic ophthalmia, scleritis, retinitis pigmentosa, immune and inflammatory components of degenerative fondus disease, inflammatory components of ocular trauma, ocular inflammation caused by infection, proliferative vitreo-retinopathies, acute ischaemic optic neuropathy, excessive scarring, e.g. following glaucoma filtration operation, immune and/or inflammation reaction against ocular implants and other immune and inflammatory-related ophthalmic diseases, inflammation associated with autoimmune diseases or conditions or disorders

where, both in the central nervous system (CNS) or in any other organ, immune and/or inflammation suppression would be beneficial, Parkinson's disease, complication and/or side effects from treatment of Parkinson's disease, AIDS-related dementia complex HIV-related encephalopathy, Devic's disease, Sydenham chorea, Alzheimer's disease and other degenerative diseases, conditions or disorders of the CNS, inflammatory components of stokes, post-polio syndrome, immune and inflammatory components of psychiatric disorders, myelitis, encephalitis, subacute sclerosing pan-encephalitis, encephalomyelitis, acute neuropathy, subacute neuropathy, chronic neuropathy, Guillaim-Barre syndrome, Sydenham chora, myasthenia gravis, pseudo-tumour cerebri, Down's Syndrome, Huntington's disease, amyotrophic lateral sclerosis, inflammatory components of CNS compression or CNS trauma or infections of the CNS, inflammatory components of muscular atrophies and dystrophies, and immune and inflammatory related diseases, conditions or disorders of the central and peripheral nervous systems, post-traumatic inflammation, septic shock, infectious diseases, inflammatory complications or side effects of surgery, bone marrow transplantation or other transplantation complications and/or side effects, inflammatory and/or immune complications and side effects of gene therapy, e.g. due to infection with a viral carrier, or inflammation associated with AIDS, to suppress or inhibit a humoral and/or cellular immune response, to treat or ameliorate monocyte or leukocyte proliferative diseases, e.g. leukaemia, by reducing the amount of monocytes or lymphocytes, for the prevention and/or treatment of graft rejection in cases of transplantation of natural or artificial cells, tissue and organs such as cornea, bone marrow, organs, lenses, pacemakers, natural or artificial skin tissue.

INTRODUCTION TO THE EXAMPLES SECTION AND THE FIGURES

The present invention will now be described only by way of example in which reference is made to the following Figures:

Figure 1 shows an MLV-based transduction method using a Cre/LoxP system as described by Vanin *et al ibid* (1997);

Figure 2 shows an EIAV-based transduction method using a Cre/Lox system;

Figure 3 shows an MLV SIN vector construct transduction method with an EIAV/HIV genome insertion using a Cre/Lox system;

Figure 4 shows an MLV-based transduction method with HRE 3'LTR using a Cre/Lox P system;

Figure 5 shows an MLV-based transduction method for MLV SIN vector production using a Cre/Lox P system;

Figure 6 shows an MLV-based transduction method with integration of a complete second genome construct using a Cre/LoxP system;

Figure 7 shows the basis molecular organisation of an RNA genome and a proviral DNA genome;

Figure 8 shows a schematic diagram of pTrap2 and pONY8z-loxP plasmids;

Figure 9 shows an overall summary of the recombinase method;

Figure 10a shows a FACS analysis of EV1 packaging cells prior to transduction with Trap2 vector;

Figure 10b shows FACS analysis of EV1 packaging cell line transduced with Trap2 at an MOI of 0.3. A 5% top slice of the highest expressers was carried out;

Figure 11 shows a validation of the method for quantitation of GFP mRNA, relative to β-actin. A titration of the total RNA from EV1 clone A was used. The difference in Ct values between the two assays is shown on the y axis. The magnitude of the gradient must be <0.1 for the method to be valid. The gradient is 0.077, so the method is suitable;

Figure 12 shows the quantitation of GFP mRNA relative to control β-actin mRNA. EV2 TD cells are transduced with Trap2 at an MOI of 0.3 and are the calibrator sample with the ratio designated 1.0;

Figure 13 shows FACS analysis of EV1 clone A:

Figure 13A shows original GFP expression of the clone;

Figure 13B shows GFP expression 7 days after transfection with Cre recombinase (pBS185). Excision frequency is 64%;

Figure 13C shows recombined clone 4 identified as being negative for GFP:

Figure 14 shows lacZ expression of transfected cells with and without the addition of the Cre recombinase (pBS185). Figure 14 shows EV1A4 and EV2D4 clones with and without the addition of Cre recombinase (pBS185). The efficiency of the insertion event was estimated to be about 12% by computer image analysis;

Figure 15 shows the structure of pONY8.1Z MLVHyb;

Figure 16 shows the alignment of leader and gag regions present in vectors pONY4Z, 8Z and ATG mutated 8Z vector. The latter is referred to as pONY8ZA. The sequences aligned are from the NarI site in the leader to the XbaI site between the EIAV gag sequence and the CMV promoter. Sequences in the leader are shown in italic and a space is present upstream of the position of the gag ATG; and

Figure 17 shows a schematic representation of the structure of pONY 8.3G +/- vector genome plasmids.

EXAMPLES

EXAMPLE 1

Vanin *et al* (*ibid*) describe a recombinase system whereby an initial retroviral transduction event introduces retroviral LTRs and expressed gene/s flanked by two recombinase target sites (exemplified by loxP) into a cell line. Stable transduced cell lines are selected by resistance to the antibiotic neomycin and screened for high expression of

the expressed gene(s) (see Figure 1). Such cell lines (Cell Line 1) contain retroviral insertions in integration sites that support high level expression from the retroviral genome.

The next step involves the transfection of the relevant recombinase expression construct (exemplified here by Cre recombinase) into the identified high expressing cell line. The expressed gene(s) is/are excised and a single loxP site is retained in the construct (Cell Line 2). In this instance, thymidine kinase gene (tk) is used as a negative selectible marker in combination with the drug, gancyclovir. The final step involves the re-insertion of a therapeutic or marker gene of choice into the single loxP site via a Cre-assisted mechanism. Cell lines are identified that have been successfully recombined (Cell Line 3) and they will produce retroviruses at the same titre as the parental Cell Line 1.

EXAMPLE 2

Figure 2 and Figure 3 describe the production of EIAV or HIV high titre transduced producer cell lines.

Figure 2 shows a minimal EIAV genome construct with the 3' U3 sequences replaced by a strong constitutive promoter, CMV. A reporter gene such as blasticidin resistance gene (*bsr*) is flanked by loxP sites. Virus is made in a transient system and is transduced into an EIAV producer cell line and clones identified that maximally express the blast marker gene. A line is chosen (termed Cell Line 1) and the marker gene is excised by a Cre recombinase-assisted excision event, generating Cell Line 2.

Construct B comprises two loxP sites which flank an internal expression cassette and also the native EIAV 3' LTR. Therefore, this construct is recombined into the cell line such that the 5' R and U5 sequences are inherited from the packaging cell line, whereas the 3' LTR sequences are wholly derived from the recombined construct. The 3' LTR from Cell Line 2 is present downstream of the functional EIAV genome expression construct. This CMV-R-U5 module is still transcriptionally active but expression is directed away from the EIAV genome.

Figure 3 shows a further aspect of the invention. Construct C is based on an MLV SIN vector, with a deletion in the 3' U3 sequences. The cassette includes an internal CMV promoter linked to EIAV R and U5 sequences. This is followed by a blasticidin resistance gene (*bsr*) flanked by two loxP sites. Virus is made in a transient transfection system and the genome is transduced into a packaging line. Blast-resistant clones are identified and the highest expressing line is chosen for further analysis. This line is transfected with Cre recombinase and the blast gene is excised. The last step involves the insertion of construct B into the single loxP site. Once again, a complete EIAV 3' LTR is introduced into the producer cell line. This leads to a CMV-driven EIAV genome expression cassette with the EIAV 3' LTR still located at the 3' end of the genome. Transcriptionally quiescent MLV SIN LTRs flanks these EIAV sequences.

EXAMPLE 3

Figure 4 shows an additional aspect of the invention. Construct D is an MLV-based vector with a CMV promoter in the 3' LTR in place of the U3 sequences. Virus is made in a transient system and is transduced into a packaging cell line as described previously. The neo and TK genes are excised by the action of Cre recombinase and construct E is recombined into the single loxP target sequence. The modified MLV 3' LTR including the HRE or similarly regulated system is transferred into the packaging cell line by the recombinase mechanism. Therefore, the 5' R and U5 sequences are inherited from the producer cell line whereas the therapeutic and marker gene/s and regulated 3' LTR is inherited from construct E. The final producer cell line is constitutively driven by the 5' CMV promoter and will produce high titre retroviral vectors which are regulated in the transduced target cells. This approach avoids the derivation of low titre transfected producer cell lines or the use of hypoxic conditions or chemical mimics for production from traditionally derived transduced producer lines.

EXAMPLE 4

Figure 5 shows yet another aspect of the invention. Construct D is an MLV-based vector with a CMV promoter in the 3' LTR as previously described. The same process is carried

out as shown in Figure 4 until the final recombination is performed. Construct F contains a deletion in U3 sequences in the 3' LTR and an internal expression cassette comprising a promoter and gene sequences. The final cell line containing the Cre-mediated recombination will be CMV-driven and will constitutively produce high titre MLV SIN vectors. Previously, SIN vectors have not been amenable to production by stable cell line producer technology. Instead they have been prepared using transfection-based transient expression systems.

EXAMPLE 5

Figure 6 shows an MLV-based transduction method with integration of complete second genome construct by Cre/LoxP system. In this approach, construct 1 is called TRAP1) is an MLV vector construct containing an internal CMV promoter operably linked to a marker gene (a truncated form of the human low affinity nerve growth factor receptor, called LNGFR). The enhancer elements in the 3' U3 sequence have been excised and replaced by a 34bp loxP site. Virus stocks are prepared in a transient system and the TRAP1 genome is stably transduced into packaging cell lines.

The modified 3'U3 sequences, including the lox P sequence, is copied from the 3'LTR position to the 5'LTR, such that there is little 5' promoter activity. Cell lines are screened for high levels of expression of LNGFR protein by fluorescent activated cell sorter (FACS) analysis and clonal lines are derived by standard techniques. A Cre recombinase expression plasmid is transfected into the derived cell line to excise all sequences between the two loxP sites. Next, cells are negatively selected by FACS for absence of LNGFR expression and clonal lines are derived by standard techniques. Construct 2 in this example comprises a complete HIV or EIAV or also MLV retroviral genome, which is flanked by two minimal 34bp loxP recombinase sites. A strong constitutive promoter such as CMV directs transcription of the genome. On transfection of plasmid 2 and Cre expression plasmid, the complete lentivirus vector or MLV vector genome is inserted in the producer cell line. These sequences are flanked to the 5' by a small portion of MLV U3 sequence and a loxP site and to the 3' by the second loxP site, enhancerless-U3 sequences, R and U5 derived from the MLV construct 1.

Derivation of Plasmid TRAP1 (Figure 6 - Construct 1)

Oligonucleotides VSAT129 and VSAT130 were synthesised which correspond to the minimal 34bp loxP sites and contain a 5' overhang for NheI and a 3' overhang for XbaI. The sequences 5' to 3' are as follows: VSAT129 (CTAGCATAACTTCGTATA ATGTATGCTATACGAAGTTATT) (SEQ ID No 49) and VSAT130 (CTAGAATAACTTCGTATAGC ATACATTATACGAAGTTATG) (SEQ ID No 50). The two oligonucleotides were treated with T4 polynucleotide kinase and were heated to 95°C for 5 minutes, before gradual cooling to room temperature. The annealed and kinased oligos were ligated to a 2,830 bp NheI/XbaI fragment from LTR plasmid (SEQ ID No 59). Fragments were ligated and correct clones of LTRloxP were identified by sequence analysis. Plasmid LTRloxP was then digested with NheI and ScaI and a 2,185bp fragment was prepared for following cloning steps.

Plasmids TRAP1 and TRAP1G were derived from LTRloxP and the MLV genome CGCLNGFR (encodes GFP and LNGFR from an internal CMV promoter – see SEQ ID No 57). However, the GFP gene was excised by EcoRI/BsmI digestion and the 6,796bp fragment was filled in by T4 DNA polymerase and re-ligated, in order to generate plasmid CXCLNGFR. Plasmid TRAP1 was generated by ligation of a 2,185bp NheI/ScaI fragment from LTRloxP (see SEQ ID No 58) to a 4,426bp NheI/ScaI fragment from CXCLNGFR. Plasmid TRAP1G was generated by ligation of a 2,185bp NheI/ScaI fragment from LTRloxP to a 5,179bp NheI/ScaI fragment from CGCLNGFR.

Derivation of Plasmid pONY8z-lox (Figure 6 - Construct 2)

In this example, the retroviral genome inserted into the loxP site in Figure 6 was based on the EIAV vector genome, pONY8z (for preparation see pONY8.0Z construction below). pONY8z was cut with SnaBI and NruI, and the 4358bp fragment purified and self-ligated to form pONY8z-shuttle. This plasmid has unique 5' sites (DraIII and BglII) and unique 3' sites (PvuII and BspLUII). Oligonucleotides encoding the 34bp loxP sites were inserted with suitable base pair overhangs at the unique 5' DraIII site and then the unique 3' BspLUII, to generate plasmid pONY-8z-shuttleloxP.

Plasmid pONY8z-loxP was made as follows. Plasmid pONY-8z-shuttleloxP was digested with BsrG I and NspV, and the 3670bp fragment was purified as the vector fragment. The insert for ligation to this fragment was derived from pONY8z by partial digestion with BsrGI (two sites) followed by digestion with NspV. A 7,328bp fragment was purified and ligated to the 3670bp fragment described above.

The Cre recombinase plasmid as used in this system is pBS185 (Gibco).

EXAMPLE 6

We constructed an MLV self-inactivating (SIN) vector called pTrap2 (see SEQ ID No 56) by replacing the 3' U3 NheI-XbaI fragment with a 34-bp loxP sequence. The vector transcribes the marker gene GFP from an internal CMV promoter. Trap2 vector was used to transduce EIAV packaging cell lines EV1 and EV2. The EV cell lines are based on human TE671 cells and express EIAV gag/pol proteins and VSV-G envelope, regulated by a temperature-sensitive switch. High expressing clones of transduced EV1 and EV2 cells were identified by FACS analysis for GFP. Individual clones expressing high levels of GFP were then selected. The GFP expression cassette was excised following transient transfection with a Cre recombinase expression plasmid. The derived cell line, EV-loxP, contains a single loxP site and minimal sequences derived from the MLV construct pTrap2. An EIAV genome was engineered to contain loxP sites flanking the entire vector genome.

This genome construct and Cre recombinase were co-transfected into EV-loxP. Stable cell lines expressing lacZ were selected by FACS and cell lines were cloned by limiting dilution. Therefore, we have introduced an entire EIAV genome expression cassette into a single loxP site. This site was previously identified by MLV transduction as highly permissive for transgene expression. A 5' CMV promoter transcribes the lentiviral genome in the producer cell line but the expression site was originally identified by MLV transduction. This method is adaptable to the generation of transduced producer cell lines for other lentiviral vector systems.

Materials and Methods

Vector construction: Plasmid pTrap2 was made as follows: A plasmid containing a single MLV LTR plasmid (LTRplasmid – SEQ ID No 59) was digested with *Nhe*I and *Xba*I and a 34 bp minimal loxP site was introduced with relevant sticky ends. This insertion step removes the MLV U3 enhancer elements which lie within the excised *Nhe*I-*Xba*I fragment. The LTR-loxP plasmid was linearised by digestion with *Nhe*I and was ligated to a 6.8kb *Nhe*I fragment from the MLV construct CZCG (See SEQ ID No 55). This construct expresses lacZ from the 5' U3 promoter and GFP from an internal CMV promoter. The resulting pTrap2 construct is shown in Figure 8.

The EIAV genome construct pONY8.0Z and pONY8.1Z were prepared as follows:

pONY8.0Z construction

pONY8.0Z was derived from pONY4.0Z (see WO 99/32646) by introducing mutations which 1) prevented expression of TAT by an 83nt deletion in the exon 2 of tat) prevented S2 ORF expression by a 51nt deletion 3) prevented REV expression by deletion of a single base within exon 1 of rev and 4) prevented expression of the N-terminal portion of gag by insertion of T in ATG start codons, thereby changing the sequence to ATTG from ATG. With respect to the wild type EIAV sequence Acc. No. U01866 these correspond to deletion of nt 5234-5316 inclusive, nt 5346-5396 inclusive and nt 5538. The insertion of T residues was after nt 526 and 543.

pONY8.1Z construction

pONY8.1Z was obtained directly from pONY8.0Z by digestion with SalI and partial digestion with SapI. Following restriction the overhanging termini of the DNA were made blunt ended by treatment with T4 DNA polymerase. The resulting DNA was then religated. This manipulation results in a deletion of sequence between the LacZ reporter gene and just upstream of the 3'PPT. The 3' border of the deletion is nt 7895 with respect to wild type EIAV, Acc. No. U01866. Thus pONY8.1Z does not contain sequences corresponding to the EIAV RREs.

Plasmid pONY8z was linearised by *Bgl*II, and a single loxP site was cloned into *Bgl*II, immediately upstream of the 5' CMV promoter, to produce pONY8z-loxP. Plasmids pONY3.2iresHYG and pHCMV-VSVG were used in the derivation of cell lines EV1 and EV2. The plasmid pONY3.2iresHYG was constructed as follows:

pONY3.2IREShyg

pONY3.IREShyg was derived from pONY3.2. pONY3.2 is a derivative of pONY3.1 in which expression of TAT and S2 are ablated by an 83nt deletion in the exon 2 of tat a 51nt deletion in S2 ORF. With respect to the wild type EIAV sequence Acc. No. U01866 these correspond to deletion of nt 5234-5316 inclusive and nt 5346-5396 inclusive. This fragment was introduced into the expression vector pHORSE IRES hyg which was made as follows. pHORSE (see WO 99/32646) was cut with SnaBI and NotI which excises a fragment running from the CMV promoter through EIAV gag/pol and introduced into pIRES1hyg (Clontech) digested with the same enzymes. This plasmid was then cut with Sse8387I and BstEII and then ligated with the Sse8387I to BstEII fragment from pONY3.2. The sequence of the plasmid is set out in SEQ ID No 51.

Virus Production

Transient MLV vector preparations pseudotyped with RD114 cat endogenous envelope were made as described previously (Soneoka et al., 1995). EIAV vector was harvested from confluent monolayers following 3 days induction of VSV-G expression at 32°C. MLV vector preparations were titred in triplicate on HT1080 fibrosarcoma cells. EIAV vector preparations were titred by GFP and lacZ on D17 dog osteosarcoma cells.

Flow cytometry of b-galactosidase and GFP activity:

$1.5x10^5$ cells from a 12-well plate were analysed for lacZ expression using the FluoReporter lacZ Flow Cytometry kit (Molecular Probes). GFP expression was also directly assessed using the FACSCalibur flow cytometer (Beckton Dickinson).

Transfection methods

Calcium phosphate transfections were carried out using the Profection kit (Promega) according to manufacturer's instructions.

Results

Figure 8 shows a schematic diagram of pTrap2 and pONY8z-loxP, plasmids used in this study.

Introduction of Trap2 genome into EV1 and EV2

An overall summary of the process described here is given in Figure 9. Trap2 MLV vector was made in a transient system with the amphotropic 4070A envelope. It gave a GFP titre of $1.7x10^6$ T.U. per ml. Trap2 vector however also gave a lacZ titre of $9.4x10^5$ T.U. per ml. This shows that replacement of the *NheI-XbaI* fragment from the MLV U3 region with loxP does not completely inactivate the MLV U3 promoter. Therefore Trap2, as constructed, is a partial SIN vector.

EV1 and EV2 cells were transduced with Trap2 vector at a multiplicity of infection (MOI) of 0.3. This was done to insert single copies of the MLV genome into the packaging lines.

Derivation of high expressers of GFP marker gene

Transduced EV1 and EV2 cells were analysed by FACS (see Figure 10) and the top 5% of GFP expressing cells were sorted and expanded. Clonal lines were derived by limiting dilution and four clones of EV1 and EV2 were chosen by visual inspection.

A quantitative TaqMan RT-PCR reaction was established in order to identify which of the four clones of EV1 and EV2 were the highest expressors of GFP mRNA. Total RNA was analysed by RT-PCR for GFP and β-actin. Quantitation was calculated by direct comparison of the Ct values (Cycle threshold). This was possible as it was proved that the two individual RT-PCR reactions are of similar efficiency (see Figure 11). By identifying an

optimal chromosomal location for GFP transgene expression. we can ensure that the inserted loxP site will be highly permissive for expression of an inserted lentiviral genome construct.

Figure 5 shows the n-fold difference in GFP : β-actin ratio for clones EV1 A to D and EV2 A to D. All ratios are defined relative to a calibrator sample, defined as a ratio of 1.0. The calibrator sample used was RNA from EV2 cells transduced with Trap2 at an MOI of 0.3.

This identified the best expressing lines as:

- EV1 clone A - GFP : β-actin ratio is 22.8
- EV2 clone D - GFP : β-actin ratio is 18.6

These two lines were carried forward for further study.

Excision of internal expression cassette by Cre recombinase

The process of retroviral integration copies the loxP-containing modified 3' U3 to the 5' position. Therefore, one can excise the majority of the MLV Trap2 integration by the action of Cre recombinase. This will leave a single modified LTR, suitable for lentiviral genome integration.

EV1 clone A and EV2 clone D were transfected by the Cre expression plasmid pBS185 (Life Technologies). After one week, the cells were analysed for GFP by FACS (see Figure 13) to determine the excision frequency. This was measured at 20-70% in all lines.

Recombined clones were identified by limit dilute cloning cells and checking by microscope and FACS for loss of GFP expression.

Insertion of EIAV genome into loxP site

Plasmid pONY8x-loxP and pBS185 were co-transfected using Fugene into EV1 clone A (excised) and EV2 clone D (excised). A control transfection of pONY8z-loxP in the absence of pBS185 was also carried out.

Figure 14 shows lacZ expression of transfected cells withand without the addition of Cre recombinase (pBS185). The efficiency of the insertion event was estmated to be ~12% by computer image analysis.

We analysed cells for lacZ expression by FACS using the FluoReporter lacZ Flow Cytometry kit. The top 5% of lacZ positive cells were sorted by FACS and clones were derived by limiting dilution. In total, 12 clones of EV1/A/pONY8z-loxP were derived and 13 clones of EV2/D/pONY8z-loxP.

EXAMPLE 7

Construction of EIAV vectors with LTR driven open reading frames

The EIAV vector configurations described previously utilise a single promoter - transgene cassette located internally in the vector. For example in pONY8Z the promoter-transgene cassette is CMV-LacZ. However for some uses it would be advantageous to have the option of expressing a gene from the 5'LTR promoter as well. For example a marker gene such as green fluorescent protein (GFP), a resistance marker such as neomycin phosphotransferase (neo) or another protein or a biologically active entity such as a ribozyme. Previous experiments have shown that the EIAV LTR is weakly active in human cells in the absence of EIAV tat. However the transcriptional activity of the LTR can be increased by replacement of the EIAV U3 region with the MLV U3 region or the CMV promoter. This is achieved by introducing these alterations in the 3'LTRs of the vector plasmids. As a result of the replicative strategy of retroviruses the modified 3'LTR becomes positioned at the 5'end of the integrated vector and can thus drive expression of a gene placed downstream of the gag region. To ensure optimal levels of expression there should preferably be no ATG start codons prior to the start codon of the gene to be expressed. In pONY8Z the ATG start codon of gag and the next ATG downstream were mutated to ATTG in order to ablate expression of the

aminoterminal portion of gag present in the vectors, however there are 7 other ATG codons further downstream of these, within gag, from which translation might be initiated.

Described below are the replacement of the U3 region of EIAV with MLV or CMV promoters and the mutation of ATG codons in the gag region

Replacement of the EIAV U3 region with MLV U3 or CMV promoters

The MLV U3 region was introduced into pONY8Z vector by replacement of the 3'LTR with a synthetic MLV/EIAV LTR made by the overlapping PCR technique, using the following primers and templates.
The EIAV PPT/U3 sequence was amplified from pONY8.1Z using primers:

KM001: CAAAGCATGCCTGCAGGAATTCG (SEQ ID No 1)

and

KM003:
GCCAAACCTACAGGTGGGGTCTTTCATTATAAAACCCCTCATAAAAACCCCAC AG (SEQ ID No 2)

to give the following product:

CAAAGCATGCCTGCAGGAATTCGATATCAAGCTTATCGATACCGTCGAATTG GAAGAGCTTTAAATCCTGGCACATCTCATGTATCAATGCCTCAGTATGTTTAG AAAAACAAGGGGGGAACTGTGGGGTTTTTATGAGGGGTTTTATAATGAAAGA CCCCACCTGTAGGTTTGGC (SEQ ID No 3)

The MLV U3 region was amplified from pHIT111 (Soneoka et al., (1995) Nucleic Acids Res. 23, 628-633) using KM004:

CTGTGGGGTTTTTATGAGGGGTTTTATAATGAAAGACCCCACCTGTAGGTTTG GC (SEQ ID No 4)

and

KM005:

GAAGGGACTCAGACCGCAGAATCTGAGTGCCCCCCGAGTGAGGGGTTGTGGG CTCT (SEQ ID No 5) to give the following product:

CTGTGGGGTTTTTATGAGGGGTTTTATAATGAAAGACCCCACCTGTAGGTTTGGCAAGCTAGCT TAAGTAACGCCATTTTGCAAGGCATGGAAAAATACATAACTGAGAATAGAGAAGTTCAGATC AAGGTCAGGAACAGATGGAACAGCTGAATATGGGCCAAACAGGATATCTGTGGTAAGCAGTT CCTGCCCCGGCTCAGGGCCAAGAACAGATGGAACAGCTGAATATGGGCCAAACAGGATATCT GTGGTAAGCAGTTCCTGCCCCGGCTCAGGGCCAAGAACAGATGGTCCCCAGATGCGGTCCAGC CCTCAGCAGTTTCTAGAGAACCATCAGATGTTTCCAGGGTGCCCCAAGGACCTGAAATGACCC TGTGCCTTATTTGAACTAACCAATCAGTTCGCTTCTCGCTTCTGTTCGCGCGCTTCTGCTCCCCG AGCTCAATAAAAGAGCCCACAACCCCTCACTCGGGGGGCACTCAGATTCTGCGGTCTGAGTCC CTTC (SEQ ID No 6)

The MLV U3/EIAV R/U5 was amplified from pONY8.1Z using primers

KM002: GAGCGCAGCGAGTCAGTGAGCGAG (SEQ ID No 7) and

KM006:

AGAGCCCACAACCCCTCACTCGGGGGGCACTCAGATTCTGCGGTCTGAGTCC CTTC (SEQ ID No 8)

to give the following product:

AGAGCCCACAACCCCTCACTCGGGGGGCACTCAGATTCTGCGGTCTGAGTCCCTTCTCTGCTG GGCTGAAAAGGCCTTTGTAATAAATATAATTCTCTACTCAGTCCCTGTCTCTAGTTTGTCTGTT CGAGATCCTACAGAGCTCATGCCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTG TTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGC CTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAAC

CTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGG
CGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTC (SEQ ID No 9)

The PCR products described above were purified and then used as templates in new PCR reactions to link them together to obtain a 992bp product. The final product contains two SapI sites which flank the hybrid LTR. These allow introduction of the PCR product into the corresponding SapI sites present in the pONY8Z or pONY8.1Z vector plasmid, thereby creating pONY8Z MLVHyb and pONY8.1 MLVHyb. The sequence of the hybrid LTR in these plasmids was confirmed by sequencing. The titres obtained from the vectos in transient transfection assays are shown in Table 1. The titres were very similar to the titres from the parental construct pONY8Z and pONY8.1Z indicating that replacement of the EIAV U3 region with that of MLV had little or no detrimental effect on the infectious cycle of the vectors.

Table 1. Titres obtained from MLV hybrid LTR vector plasmids

vector plasmid	#titre (l.f.u./ml)
pONY8Z	3×10^5
pONY8Z MLVHyb	1×10^5
pONY8.1Z	6×10^4
pONY8.1Z MLVHyb	2×10^4

Titre was measured on D17 cells and is expressed as LacZ forming units/ml (l.f.u./ml). Transfections were carried out in 293T cells using the vector plasmid shown and pRV67 (VSV-G expression plasmid), and pONY3.1 (EIAV gag/pol expression plasmid).

The structure of pONY8.1Z MLVHyb is shown in Figure 15 and the sequence of this plasmid is shown as SEQ ID No 10.

The EIAV promoter was also replaced by the human cytomegalovirus (CMV) promoter using a similar strategy. The primers and templates were the same except that KM003 was replaced by KM008:

GGCCATCGTGCCTCCCCACTCCTGCAGTTATAAAACCCCTCATAAAAACCCCA CAG (SEQ ID No 11)

KM004 was replaced by KM009:

CTGTGGGGTTTTTATGAGGGGTTTTATAAACTGCAGGAGTGGGGAGGCACGA TGGCC (SEQ ID No 12)

KM005 was replaced by KM010:

GAAGGGACTCAGACCGCAGAATCTGAGTGCCCGGTTCACTAAACGAGCTCTG CTTATATAGACC (SEQ ID No 13) and

KM006 was replaced by KM011:

GGTCTATATAAGCAGAGCTCGTTTAGTGAACCGGGCACTCAGATTCTGCG GTCTGAGTCCCTTC (SEQ ID No 14)

The template for the PCR reaction with primers KM009 and KM010 was pONY2.1LacZ. This plasmid contains a single CMV promoter. The combined PCR product of 1319 bp was digested with SapI and introduced into the pONY8Z or pONY8.1Z backbone as described above for pONY8Z MLVHyb.

Mutation of remaining ATG codons in the gag of pONY8Z to ATTG

The alignment of the sequence of the leader and gag region present in vectors pONY4Z (an earlier generation EIAV vector), pONY8Z and a derivative of pONY8Z in which the 7 remaining ATG codons are mutated to ATTG is shown in Figure 16. These mutations were created by PCR mutagenesis as follows. The template for the PCR reactions was pONY8Z and the primers were:

F1: CGAGATCCTACAGTTGGCGCCCGAACAG (SEQ ID No 15);

R1:GAGTTACAATCTTCCAGCAATGGAATGACAATCCCTCAGCTGCCAGTCCTT TTCTTTTACAAAGTTGGTATCAATGAAATAAGTCTACTAGACTTAGC (SEQ ID No 16);

F2:TTCCATTGCTGGAAGATTGTAACTCAGACGCTGTCAGGACAAGAAAGAGA GGCCTTTGAAAGAACATTGGTGGGCAATTTCTGCTGTAAAGATTG (SEQ ID No 17);

R2:CAATATTTCGCTCTTAGGAGCTGGAATGATGCCTTTCCAATCTACTACAAT TATTAATCTGGAGGCCCAATCTTTACAGCAGAAATTGCCCACCAATG (SEQ ID No 18);

R3:CCACTAGTTCTAGAGATATTCTTCAGAGGGCTCAGACTGCTTTTTATTAGC AGTCTTCTTTTCAATATTTCGCTCTTAGGAGC (SEQ ID No 19)

In the first stage of construction two PCR reactions were set up with primer pairs F1/R1 and F2/R2, respectively. These were purified and then used in a second 'overlapping' reaction in which primers F1 and R3 were added after 10 cycles. This procedure results in a 552bp PCR product (SEQ ID No 20):

CGAGATCCTACAGTTGGCGCCCGAACAGGGACCTGAGAGGGGCGCAGACCCTACCTGTTGAA CCTGGCTGATCGTAGGATCCCCGGGACAGCAGAGGAGAACTTACAGAAGTCTTCTGGAGGTGT TCCTGGCCAGAACACAGGAGGACAGGTAAGATTGGGAGACCCTTTGACATTGGAGCAAGGCG CTCAAGAAGTTAGAGAAGGTGACGGTACAAGGGTCTCAGAAATTAACTACTGGTAACTGTAAT TGGGCGCTAAGTCTAGTAGACTTATTTCATTGATACCAACTTTGTAAAAGAAAAGGACTGGCA GCTGAGGGATTGTCATTCCATTGCTGGAAGATTGTAACTCAGACGCTGTCAGGACAAGAAAGA GAGGCCTTTGAAAGAACATTGGTGGGCAATTTCTGCTGTAAAGATTGGGCCTCCAGATTAATA ATTGTAGTAGATTGGAAAGGCATCATTCCAGCTCCTAAGAGCGAAATATTGAAAAGAAGACTG CTAATAAAAAGCAGTCTGAGCCCTCTGAAGAATATCTCTAGAACTAGTGG

This was digested with NarI and XbaI and ligated into pONY8Z. pONY8Z MLVHyb and pONY8Z CMVHyb. which had been prepared for ligation by digestion with the same enzymes. These plasmids were designated pONY8ZA or pONY8ZA MLVHyb and pONY8ZA CMVHyb. The sequence for pONY8ZA CMVHyb is provided in SEQ ID No 52. These plasmids have a unique XbaI site into which can be inserted genes such as

GFP or neomycin phosphotransferase or other biologically active entity. This use of th site is demonstrated for GFP. The GFP ORF was obtained from pEGFP-1 (Clontech) by digestion with SmaI and XbaI, and then the ends filled in by treatment with T4 DNA polymerase. This fragment was then ligated into pONY8ZA or pONY8ZA MLVHyb and pONY8ZA CMVHyb prepared for ligation by digestion with XbaI and subsequent filling in with T4DNA polymerase. The resulting vector plasmids were called pONY8GZA or pONY8GZA MLVHyb and pONY8GZA CMVHyb. Other genes can be inserted at this site by manipulations apparent to those skilled in the art.

Creation of EIAV vector genomes containing loxP sites in their LTR's

The time taken to construct producer cell lines for EIAV vectors would be greatly reduced if it was possible to 1) locate and 2) reutilise a site in the host cell chromosome which was particularly favourable for high levels of transcription of the vector genome. In outline, this can be achieved by engineering loxP sites in the 3'LTR of EIAV vectors, transduction of the packaging cell line with vectors which carry loxP and hybrid LTRs, selection of cells which express the highest levels of vector genome and exchange of the test EIAV vector genome for the vector genome of choice using the cre/loxP recombination system.

The proposed scheme was evaluated using a derivative of pONY8GZA CMVHyb in which a loxP site was introduced into the PstI site between the EIAV sequences (required for efficient integration) and the CMV promoter in the 3'LTR. After transduction the integrated vector will thus have a loxP-CMV cassette located in the 5'LTR and 3'LTR's and therefore full length transcripts of the vector genome will be driven by the CMV promoter, which is a powerful promoter. pONY8GZA CMVHyb contains many PstI sites hence it was modified to allow insertion of the loxP site by digestion with XbaI and NheI and religation to create the subclone, pONY CMVHyb. This plasmid has a unique PstI site in the hybrid LTR. The loxP site was inserted into this site using two complimentary oligonucleotides which when annealed formed PstI-compatible termini. These were termed loxP POS

PSTI [GATAAC TTCGTATAATGTATGCTATACGAAGTTATCTGCA] (SEQ ID No 21)] and

loxP NEG PstI [GATAACTTCGTATAGCATACATTATACGAAGTTATCTGCA] (SEQ ID No 22)

The sequence and orientation of the loxP site was confirmed by DNA sequencing and the plasmid called pONY CMVHyb loxP. The central part of the vector genome was then reintroduced into this subclone by transfer of the NotI-BstEII fragment from pONY8GZA CMVHyb into pONY CMVHyb cut the same way. The resulting vector was termed pONY8GZA CMVHyb loxP.

Two routes for construction of a producer cell line are available using pONY8GZA CMVHyb loxP. The plasmid can be introduced into a packaging cell line by transfection or vector particles can be made using the 293T and these used to transduce the packaging cell line. Since the vector is derived from EIAV, rather than MLV, it is able to transduce non-dividing cells or slowly dividing cells. In this situation it has been hypothesised that integrations occur at chromosomal sites that are constitutively open; that is, are likely to be sites at which high levels of transcription will be maintained for extended periods. This may be important for the long term usefulness of the producer cell line and thus represents an advantage of strategy using transduction.

Producer cell lines were made by transfection or transduction of a TE671-derived cell line (EV11E) which has stably integrated copies of VSV-G and the synthetic EIAV gag/pol under the control of CMV promoters. Prior to transfection with pONY8GZA CMVHyb loxP it was linearised by digestion with AhdI. Seven days following transfection or transduction cells expressing the highest levels of GFP were selected by FACS and then cloned by limiting dilution. A number of clones were analysed for levels of full length vector RNA using Taqman technology based assays in order to confirm the hypothesis that the highest level of GFP expression correlates with the highest levels of vector RNA.

The cell line which expressed the highest level of RNA was then tested for production of transducing vector particles 5 days after changing the temperature of incubation from 37C

to 32°C. At 32°C, VSV-G expression is induced however maximal levels of VSV-G are only obtained after 5 days at the permissive temperature (see WO 00/52188). The cell line producing the highest titre, EV11E CMV loxP was selected for further work.

In order to exchange the vector genome with for another EIAV vector genome with a more suitable configuration for use in the clinical setting EV11EloxP cells were transfected with cre recombinase expression plasmid, pBS185 (Gibco), which results in excision of the EIAV vector between the loxP sites. This leaves a loxP-CMV promoter R-U5 sequence in the cells. Cells from which the EIAV vector genome had been excised were selected on the basis of low levels of GFP expression by FACS and assumed to be clonal on the basis of the clonality of EV11E CMV loxP. These were termed EV11EloxPΔ and used as targets for new EIAV vector genomes.

Construction of EIAV vector genomes with downstream REV expression cassettes and flanking loxP sites

The production of vector particles from minimal EIAV vectors (those which do not express EIAV REV or any other EIAV proteins) is increased by about 10-fold in the presence of EIAV REV in our 293T transient production system when the codon-optimsed EIAV gag/pol expression construct is used to drive production of vector particles as set out in Table 2. This may be improved nuclear to cytoplasmic transport of the vector genome in the presence of REV protein. Packaging/producer cell lines for EIAV vectors may be engineered to express Rev protein. One approach would be to engineer cells to express EIAV REV from an independently transfected expression cassette. However, the cassette and the vector genome may be subject to differential regulation, for example by methylation or chromosome remodelling. Such an effect may limit the useful life of such cell lines.

Table 2. Effect of REV expression on titres obtained from REV-expressing [REV+] and non-expressing [REV-] vectors

vector plasmid	gag/pol expression	#titre

	plasmids	(l.f.u./ml)
pONY4Z [REV+]	pONY3.1	$2.0\pm0.4 \times 10^6$
pONY4Z [REV+]	pE SYN GP	$0.9\pm0.2 \times 10^6$
pONY8Z [REV-]	pONY3.1	$1.5\pm0.2 \times 10^6$
pONY8Z [REV-]	pE SYN GP	$1.9\pm0.6 \times 10^5$

[#] Titre was measured on D17 cells and is expressed as LacZ forming units/ml (l.f.u./ml). Transfections were carried out in 293T cells using the vector plasmid and gag/pol expression plasmid shown and pRV67 (VSV-G expression plasmid) (See WO 00/52188).

REV+ and REV- reflect the rev expression status of the vectors. REV+ reflects vectors which express the REV protein. REV- reflects expression vectors which do not express the REV protein.

pESYNGP

The gag/pol expression plasmid shown in Figure called called pESYNGP was constructed as follows: The codon-optimised EIAV gag/pol ORF was synthesised by Operon Technologies Inc., Alameda and supplied in a proprietary plasmid backbone, GeneOp. The complete fragment synthesised included sequences flanking the EIAV gag/pol ORF: tctagaGAATTCGCCACC**ATG- EIAV gag/pol- UGA**ACCCGGGgcggccgc (SEQ ID No 44). The ATG start and UGA stop codons are shown in bold. XbaI and NotI sites are in lower case. These were used to transfer the gag/pol ORF from GeneOp into pCIneo (Promega) using the NheI and NotI sites in the latter.

pESDSYNGP

An alternative expression plasmid for expression of the synthetic EIAV gag/pol could also be used. It is called pESDSYNGP and its construction is described as follows: ESDSYNGP was made from pESYNGP by exchange of the 306bp EcoRI-NheI fragment, from just upstream of the start codon for gag/pol to approximately 300 base pairs inside

the gag/pol ORF with a 308bp EcoRI-NheI fragment derived by digestion of a PCR made using pESYNGP as template and using the following primers: SD FOR [GGCTAGAGAATTCCAGGTAAGATGGGCGATCCCCTCACCTGG] (SEQ ID No 60) and SD REV [TTGGGTACTCCTCGCTAGGTTC] (SEQ ID No 61). This manipulation replaces the Kozak concensus sequence upstream of the ATG in pESYNGP with the splice donor found in EIAV. The sequence between the EcoRI site and the ATG of gag/pol is thus CAGGTAAG (SEQ ID No 62).

The sequences for pESYNGP (SEQ ID No 53) and pESDSYNGP (SEQ ID No 54) are provided.

Packaging/Producer cells may be engineered by physically linking the genome and EIAV REV expression cassettes. In this way stable transfectants may be generated which contain the vector genome and the EIAV REV expression cassette in the same chromatin environment. This manipulation may ensure that the relative levels of transcription of the vector genome and the REV expression cassette are maintained leading to an increased duration of vector production from the producer cells.

Previous work has suggested that optimisation of the level of REV may be required with respect to the level of vector genome (see WO 98/17815). We have examined the levels of vector production in a transient system in which several different promoters are used to drive REV expression in order to determine which vector genome-rev expression cassette is optimal for use in constructing producer cell lines. The highest titres were obtained with FB29 and PGK promoters driving REV expression.

The following describes the construction of EIAV vector genomes plasmids in which there is a downstream expression cassette for synthetic EIAV REV protein. The promoters tested were FB29, PGK, TK, CMV, SV40 and RSV. In addition the loxP sites were engineered into the vector plasmid backbone in such a way that the genome and introduced promoter-REV expression plasmid was flanked. In this way, the complete vector-REV cassette can be recombined into loxP sites in the target cell.

The complete construction of the FB29 and PGK containing plasmids is described here. The REV expression construct was inserted in the both orientations with respect to the EIAV vector genome. Plasmids in which the FB29 or PGK promoters drive REV expression are being utilised for construction of stable producer cell lines.

Construction of plasmids

In the first step of construction an SfiI site was inserted downstream of the EIAV vector sequence. This site is the insertion site for the promoter REV cassettes. The construction was made as follows. pONY8Z was digested with EheI and NruI, the ends were blunted by treatment with T4 DNA polymerase and religated. The resulting plasmid, pONY8Z delta, is thus deleted with respect to the leader, gag, reporter cassette and most of the Rev/RRE regions.

pONY8Z delta was mutated to contain loxP sites inserted in the DraII site immediately to the 5'of the CMV promoter and in the BspLU11I site to the 3' of the vector genome. The loxP sites were inserted using complementary nucleotide pairs which when annealed had overhanging termini suitable for cloning into these sites and were inserted in two steps of cloning. The oligonucleotides for insertion into the DraIII site were

VSAT 158: [GTGATAACTTCGTATAATGTATGCTATACGAAGTTATCACTAC] (SEQ ID No 23)

and

VSAT 155 [GTGATAACTTCGTATAGCATACATTATACGAAGTTATCACGTA] (SEQ ID No 24)

For the BspLU11I they were:

VSAT 156 [CATGTATAACTTCGTATAATGTATGCTATACGAAGTTATA] (SEQ ID No 25) and

VSAT 157 [CATGTATAACTTCGTATAGCATACATTATACGAAGTTATA] (SEQ ID No 26)

Plasmids were selected in which the orientation of the loxP at both sites were the same and the same as the EIAV vector genome. The modified plasmid was called pONY8Z delta 2xloxP.

pONY8Z delta 2xloxP has a unique PvuII site downstream of the deleted EIAV vector genome into which annealed complementary oligonucleotides encoding SfiI sites were inserted. The oligonucleotides were:

SFI SRFPOS [AGTAGGCCGCCTCGGCCGCCCGGGCATCA] (SEQ ID No 27) and

SFI SRF NEG [TGATGCCCGGGCGGCCGAGGCGGCCTACT] (SEQ ID No 28)

Clones which had the SfiI – SrfI sites in either orientation were selected for further work. These were called pONY8Z delta SfiI FOR and REV.

Amplification and cloning of FB29 and PGK promoters

The FB29 promoter was amplified from pRDF (Cosset FL, et al.*J Virol* 1995 Dec;69(12):7430-6) using primers:

FB29 POS [TAGCCGAGATCTCAAATTGCTTAGCCTGATAGCC] (SEQ ID No 29)and

FB29 NEG [TGCGTAGCTAGCCTCCCGGTGGTGGGTCGGTG] (SEQ ID No 30) which introduce

5'BglII and 3'NheI sites.

The PGK promoter was amplified from pPE327 using primers

PGK POS [AGCAGTAGATCTGGGGTTGGGGTTGCGCCTTT] (SEQ ID No 31) and

PGK NEG [CGTCATGCTAGCCTGGGGAGAGAGGTCGGTG] (SEQ ID No 32)

The PGK promoter sequence obtained from this plasmid was the same as the sequence of GenBank Acc. No. M11958 except that it has a single mutation: nucleotide 347 of M11958 is changed from G to A. The TK promoter and intron was amplifed from pRL-TK (Promega) with:

TK POS [TACGGAAGATCTAAATGAGTCTTCGGACCT] (SEQ ID No 33) and

TK NEG [CTCAACGCTAGCGTACTCTAGCCTTAAGAGCTG] (SEQ ID No 34)

The RSV promoter was amplifed from pREP7 (Invitrogen) with

RSV POS [TACCAGAGATCTTCTAGAGTCGACCAATTCTCATG] (SEQ ID No 35) and

RSV NEG [CATCGAGCTAGCAGCTTGGAGGTGCACACCAATG] (SEQ ID No 36) and

The SV40 promoter was amplifed from pCIneo (Promega) with:

SV40 POS [GATGGTAGATCTGCGCAGCACCATGGCCTGAA] (SEQ ID No 37) and

SV40 NEG [CTCGAAGCTAGCAGCTTTTTGCAAAAGCCTAGGC] (SEQ ID No 38)

The PCR fragments were digested with BglII and NheI and ligated into pSL1180 (Pharmacia) which had been prepared by digestion using the same enzymes. Following transformation into E.coli DNA was prepared and the sequence of the promoters checked by DNA sequencing. Clones in which the correct promoter sequence was present were used for further work and were called pSL1180-FB29, pSL1180-PGK, pSL1180-RSV, pSL1180-SV40, pSL1180-TK.

In the next step the promoter fragments were positioned to drive transcription of synthetic EIAV REV in pE syn REV. pE syn REV is a pCIneo based plasmid (Promega) which was made by introducing the EcoRI to SalI fragment from the synthetic EIAV REV plasmid into the polylinker region of the plasmid using the same sites. The synthetic

EIAV REV plasmid made by Operon contains a codon-optimised EIAV REV open reading frame flanked by EcoRI and SalI. The sequence of this fragment is shown as SEQ ID No 39.

Prior to replacement of the CMV promoter in pE syn REV it was modified as follows. The SV40 neo region was deleted by digestion with KpnI and BamHI, the ends blunted by treatment with T4 DNA polymerase and then religated. The plasmid was termed pE syn REV delta. Next SfiI sites were introduced into both the BglII site which is just 5' of the CMV promoter and DraIII site downstream of the polyA signal.

The oligonucleotides used for this were as follows:

SFI FOR BglII POS [GATCGGCCGCCTCGGCCA] (SEQ ID No 40) and

SFI FOR BglII NEG [GATCTGGCCGAGGCGGCC] (SEQ ID No 41)and

SFI FOR DRA POS [GGCCGCCTCGGCCGTA] (SEQ ID No 42) and

SFI FOR DRA NEG [GGCCGAGGCGGCCTAC] (SEQ ID No 43)

Clones in which the SfiI was located 5' of the BglII site were selected were used for further work. The plasmid obtained after this two step manipulation was termed pE syn REV delta 2xSfiI. It has the following features: 5'SfiI sites – BglII site - CMV promoter and intron – NheI site – E syn REV - polyA site – 3'SfiI site. Hence the CMV promoter can be excised by digestion with BglII and NheI and replaced with the promoter of choice obtained from the pSL1180 series of clones by digestion with the same enzymes. Construction details are included from this point for only the constructs which contained FB29 and PGK promoters, however a similar scheme was used for the other promoters, except that a partial SfiI digestion was required for transfer of the SV40-REV cassette.

Promoter fragment were obtained from pSL1180 – FB29 and pSL1180 – PGK by digestion with BglII and NheI and ligated into pE syn REV delta 2xSfiI digested with the

same enzymes. The resulting plasmids were called FB29 E SYN REV and PGK E SYN REV.

In the next stage the internal regions of pONY8G, pONY8.1G SIN MIN and pONY4G were obtained by digestion with SgfI (which has unique site in the CMV promoter driving the EIAV vector genome) and MunI (which cuts in the 3'LTR) and ligated in to pONY8Z delta SfiI FOR and REV prepared for ligation by digestion with the same enzymes. The resulting plasmids were called pONY8G SfiI FOR and REV, pONY8.1G SIN MIN SfiI FOR and REV and pONY4G SfiI FOR and REV.

In the final stage the promoter-REV cassettes were moved from FB29 E SYN REV and PGK E SYN REV into pONY8G SfiI FOR and REV, pONY8.1G SIN MIN SfiI FOR and REV and pONY4G SfiI FOR. This manipulation was achieved as follows. FB29 E syn REV, PGK E syn REV, and the vector plasmids described immediately above were digested with SfiI and ligations set up with appropriates fragments. The promoter-REV cassettes were orientated in the same or opposite orientations with respect to the EIAV vector genome in the 'FOR' and 'REV' plasmids The resulting plasmids were called pONY8.3G FB29 + or – and pONY8.3G PGK+ and –. A schematic structure of the pONY 8.3 +/- plasmids is shown in Figure 17.

The performance of these constructs was tested in relation to pONY8G in 293T transient production assays and the results are shown in Table 3.

The sequence of the *EcoR*I to *Sal*I fragment representing the codon-optimised EIAV REV open reading frame obtained from the plasmid synthesised by Operon (SEQ ID No 39)

EcoRI and SalI sites are in bold. The ATG start and UGA termination codons are underlined

GAATTCGCCACCATGGCTGAGAGCAAGGAGGCCAGGGATCAAGAGATG

ACCTCAAGGAA
GAGAGCAAAGAGGAGAAGCGCCGCAACGACTGGTGGAAGATCGACCCA
AAGGCCCCCTG
GAGGGGGACCAGTGGTGCCGCGTGCTGAGACAGTCCCTGCCCGAGGAGAAGATTCCT
AGC
CAGACCTGCATCGCCAGAAGACACCTCGGCCCCGGTCCCACCCAGCACACACCCTCC
AGA
AGGGATAGGTGGATTAGGGGCCAGATTTTGCAAGCCGAGGTCCTCCAAGAAAGGCTG
GAA
TGGAGAATTAGGGGCGTGCAACAAGCCGCTAAAGAGCTGGGAGAGGTGAATCGCGG
CATC
TGGAGGGAGCTCTACTTCCGCGAGGACCAGAGGGGCGATTTCTCCGCATGGGGAGGC
TAC
CAGAGGGCACAAGAAAGGCTGTGGGGCGAGCAGAGCAGCCCCCGCGTCTTGAGGCC
CGGA
GACTCCAAAAGACGCCGCAAACACCTGTGAAGTCGAC

Table 3

Titres obtained from a representative experiment in which the vector-REV constructs were tested by transient 293T production assay. The vector constructs were cotransfected with pE synGP, the synthetic EIAV gag/pol expression plasmid, and pRV67, VSV-G expression plasmid. Titres were measured in D17 cells.

Plasmid	Titre (g.f.u./ml)
pONY8G SfiI FOR	$1.6x10^4$
#pONY8G SfiI FOR PLUS pE syn REV	$5.2x10^5$
pONY8.3G FB29 +	$8.8x10^5$
pONY8.3G FB29 -	$7.8x10^5$
pONY8.3G PGK +	$1.2x10^6$
pONY8.3G PGK -	$1.2x10^6$
pONY8G	$9.4x10^5$

Titre was assessed on D17 cells and is expressed as green fluorescent protein cell units/ml (g.f.u./ml). Transfections were carried out with pE syn GP KOZAK and pRV67 as described previously.

* pONY8G SfiI FOR is identical to the pONY8.3 derivatives except that there is no promoter-REV expression cassette is inserted in the SfiI site

pE syn REV plasmid was also included in this transfection

pONY8G is a standard EIAV vector genome used for comparative purposes

pONY8.3G FB29 – is shown as SEQ ID No 45

pONY8.3G FB29 + is shown as SEQ ID No 46

pONY8.3GPGK – is shown as SEQ ID No 47

pONY8.3G PGK + is shown as SEQ ID No 48.

SUMMARY

Thus, in summation, the present invention provides high titer regulated retroviral vectors. These regulated retroviral vectors include lentivectors, HRE-regulated vectors and functional SIN vectors which can be produced at high titres from derived producer cell lines.

The present invention also provides a method other than retroviral transduction for the transfer of a regulated retroviral vector into a derived producer cell line. This method comprises a recombinase assisted method which allows for the production of high titer regulated retroviral vectors.

In one broad aspect, the present invention relates to the selection of cells which express high levels of a retroviral vector genome and exchange of this retroviral genome for the

vector genome of choice, preferably a regulated retroviral vector genome or a lentiviral vector genome using a cre/loxP recombination system. Thus, the present invention enables regulated retroviral vectors to be produced at high titres from transduced producer cell lines.

In another broad aspect, the present invention relates to the selection of cells which express high levels of a retroviral vector genome and exchange of this retroviral genome for the vector genome of choice, preferably a regulated retroviral vector genome or a lentiviral vector genome using a cre/loxP recombination system and a retroviral vector producton system which incorporates a REV protein production system. Thus, the present invention enables regulated retroviral vectors to be produced at high titres from transduced producer cell lines.

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.

CLAIMS

1. A method of modifying a producer cell which producer cell comprises integrated into its genome a provirus which provirus comprises one or more recombinase recognition sequences within or upstream of its 3' LTR, the method comprising:
introducing into the cell a construct comprising a 5' recombinase recognition sequence, an LTR and a 3' recombinase recognition sequence in that order, in the presence of a recombinase which is capable of acting on the recombinase recognition site(s) such that the nucleotide sequence between the 5' and 3' recombinase recognition sequences in the construct is introduced into the provirus.

2. A method according to claim 1 wherein the construct further comprises at least one nucleotide sequence of interest (NOI) between the 5' recombinase recognition sequence and the LTR, which NOI is operably linked to a transcriptional regulatory sequence.

3. A method according to claim 1 or claim 2 wherein the construct further comprises a 5'LTR and/or a packaging signal.

4. A method according to any one of claims 1 to 3 wherein the LTR is a heterologous regulatable LTR.

5. A method according to claim 4 wherein the regulatable LTR comprises an ischaemic like response element (ILRE).

6. A method according to any one of claims 1 to 3 wherein the LTR is inactive.

7. A method according to any one of the preceding claims wherein the provirus comprises an NOI encoding a selectable marker, which NOI is flanked by recombinase recognition sites

8. A method according to any one of the precedings claims wherein the provirus comprises an internal 5' LTR upstream of the recombinase site or the 5' recombinase site

where there is more than one site.

9. A method according to any one of the preceding claims wherein the U3 region of the 5' LTR is inactive.

10. A method according to any one of the preceding claims wherein the U3 region of the 5' LTR and/or the U3 region of the second internal 5'LTR comprises a heterologous promoter.

11. A method according to any one of the preceding claims wherein the provirus comprises two recombinase recognition sites and as a preliminary step, the recombinase is expressed in a host cell such that the nucleotide sequence present between the two sites is excised.

12. A method according to any one of the preceding claims wherein the producer cell is a high titre producer cell.

13. A method according to any one of the preceding claims wherein the provirus is a lentivirus.

14. A method according to claim 13 wherein the lentivirus is HIV or EIAV.

15. A method according to any one of claims 2-14 wherein the provirus further comprises a second NOI.

16. A producer cell obtainable by the method of any one of claims 1 to 15.

17. An infectious retroviral particle obtainable from the producer cell of claim 16.

18. A derived producer cell comprising integrated into its genome a retroviral vector comprising in the 5' to 3' direction a first 5' LTR; a second NOI operably linked to a second regulatable 3' LTR; and a third 3'LTR;

wherein the third 3'LTR is positioned downstream of the second regulatable 3'LTR in the producer cell.

19. A producer cell according to claim 18 wherein the first 5' LTR comprising 5'R and 5' U5 sequences is derivable from a first vector; the second NOI operably linked to a second regulatable 3' LTR is derivable from a second vector; and the third 3'LTR is derivable from the first vector.

20. A producer cell according to claim 18 or claim 19 wherein the first vector comprises a retroviral vector wherein the retroviral vector comprises a first NOI flanked by recombinase recognition sequences.

21. A producer cell according to claim 19 or claim 20 wherein the retroviral vector further comprises an internal LTR located upstream of the first NOI and downstream of a packaging signal wherein the internal LTR comprises a heterologous U3 sequence linked to heterologous R and U5 sequences.

22. A producer cell according to any one of claims 18 to 21 wherein the third 3'LTR is transcriptionally quiescent.

23. A producer cell according to claim 22 wherein the third 3' LTR comprises a deletion in the U3 sequence.

24. A producer cell according to any one of claims 18 to 23 wherein the first NOI is a selectable marker.

25. A producer cell according to claim 19 wherein the second vector comprises a second NOI operably linked to a second regulatable 3'LTR comprising at least one recombinase recognition sequence.

26. A producer cell according to 25 wherein the second regulatable 3'LTR comprises a deletion in the U3 sequences in the 3'LTR.

27. A producer cell according to claim 25 or claim 26 wherein the second NOI comprises a coding sequence operably linked to a promoter.

28. A producer cell according to claim 27 wherein the second NOI comprises a discistronic construct.

29. A producer cell according to claim 28 wherein the discistronic construct comprises a therapeutic gene, an internal ribosomal entry site (IRES) and a reporter gene.

30. A method for producing a high titre regulatable retroviral vector, the method comprising the steps of:

(i) providing a derived producer cell comprising integrated into its genome a first vector;

(ii) introducing a second vector into the derived producer cell using a recombinase assisted method;

wherein the derived producer cell comprises a retroviral vector comprising in the 5' to 3' direction a first 5' LTR; a second NOI operably linked to a second regulatable 3' LTR; and a third 3'LTR; wherein the third 3'LTR is positioned downstream of the second regulatable 3'LTR in the derived producer cell.

31. A method according to claim 30 wherein the third 3' LTR is transcriptionally active but expression is directed away from the second regulatable 3'LTR.

32. A method for introducing a second regulatable 3'LTR into a derived producer cell wherein the method comprises a recombinase assisted method.

33. A method according to claim 32 wherein the recombinase assisted method is a Cre/lox recombinase method.

34. A process for preparing a regulated retroviral vector as defined in claim 17 comprising performing the method according to any one of claims 30 to 33 and preparing a quantity of the regulated retroviral vector.

35. A regulated retroviral vector produced by the process according to claim 34.

36. A regulated retroviral vector according to claim 35 wherein the retroviral vector is capable of transducing a target site.

37. A regulated retroviral vector according to claim 36 wherein the retroviral vector is produced in sufficient amounts to effectively transduce a target site.

38. A regulated retroviral vector according to claim 36 or claim 37 wherein the target site is a cell.

39. A cell transduced with a regulated retroviral vector according to claim 38.

40. Use of a regulated retroviral vector according to any one of claims 35 to 38 in the manufacture of a pharmaceutical composition to deliver an NOI to a target site.

41. Use of a regulated retroviral vector according to any one of claims 35 to 38 in the manufacture of a medicament for diagnostic and/or therapeutic and/or medical applications.

42. Use of a recombinase assisted mechanism to introduce a regulated 3'LTR into a derived producer cell line to produce a high titre regulated retroviral vector.

43. A derived stable producer cell capable of expressing regulated retroviral vectors according to claims 35 to 38.

44 A derived stable producer cell according to claim 43 wherein the regulated retroviral vector is a high titre regulated retroviral vector.

49. A nucleic acid molecule according to any one of claims 46 to 48 wherein the LTR is a heterologous regulatable LTR.

50. A nucleic acid molecule according to any one of claims 46 to 48 wherein the LTR is transcriptionally quiescent.

51. A method and/or a producer cell substantially as described herein and with reference to the accompanying Figures.

FIG. 1

MLV-based transduction using Cre/loxP system as previously described

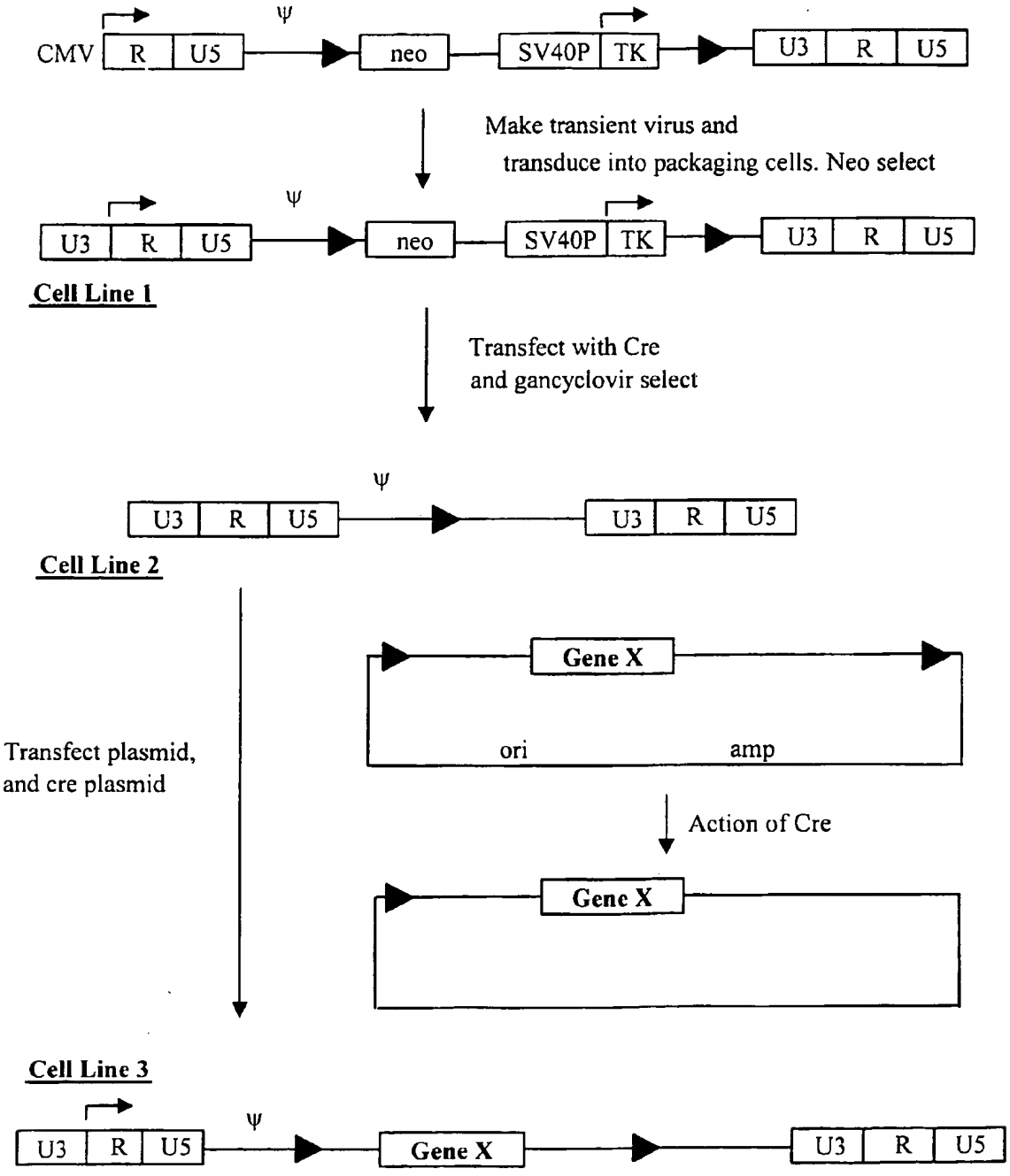


FIG. 2

EIAV-based transduction Cre/loxP system

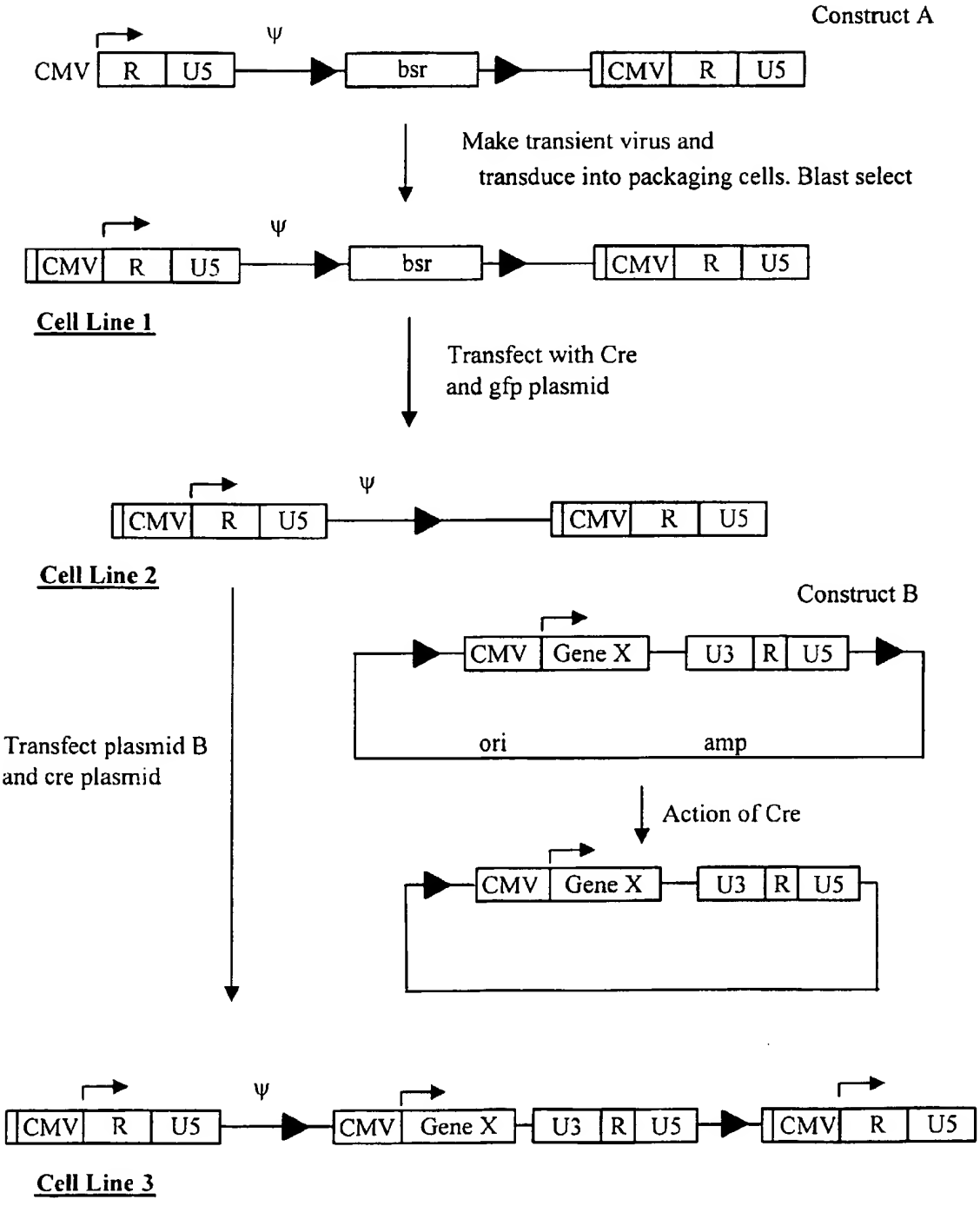


FIG. 3

MLV SIN vector approach, with EIAV components in blue

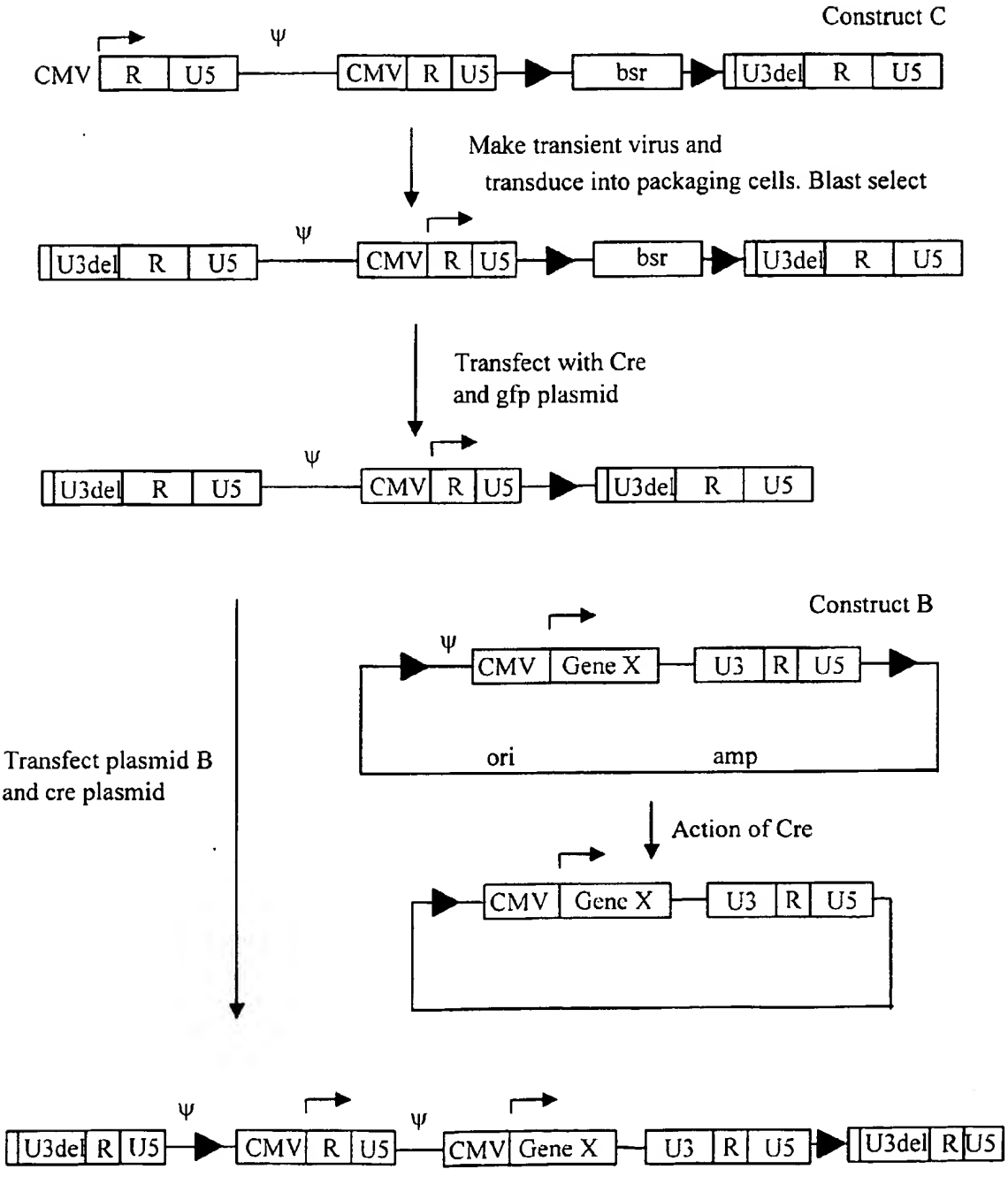


FIG. 4

MLV-based transduction with HRE 3' LTR using Cre/loxP system

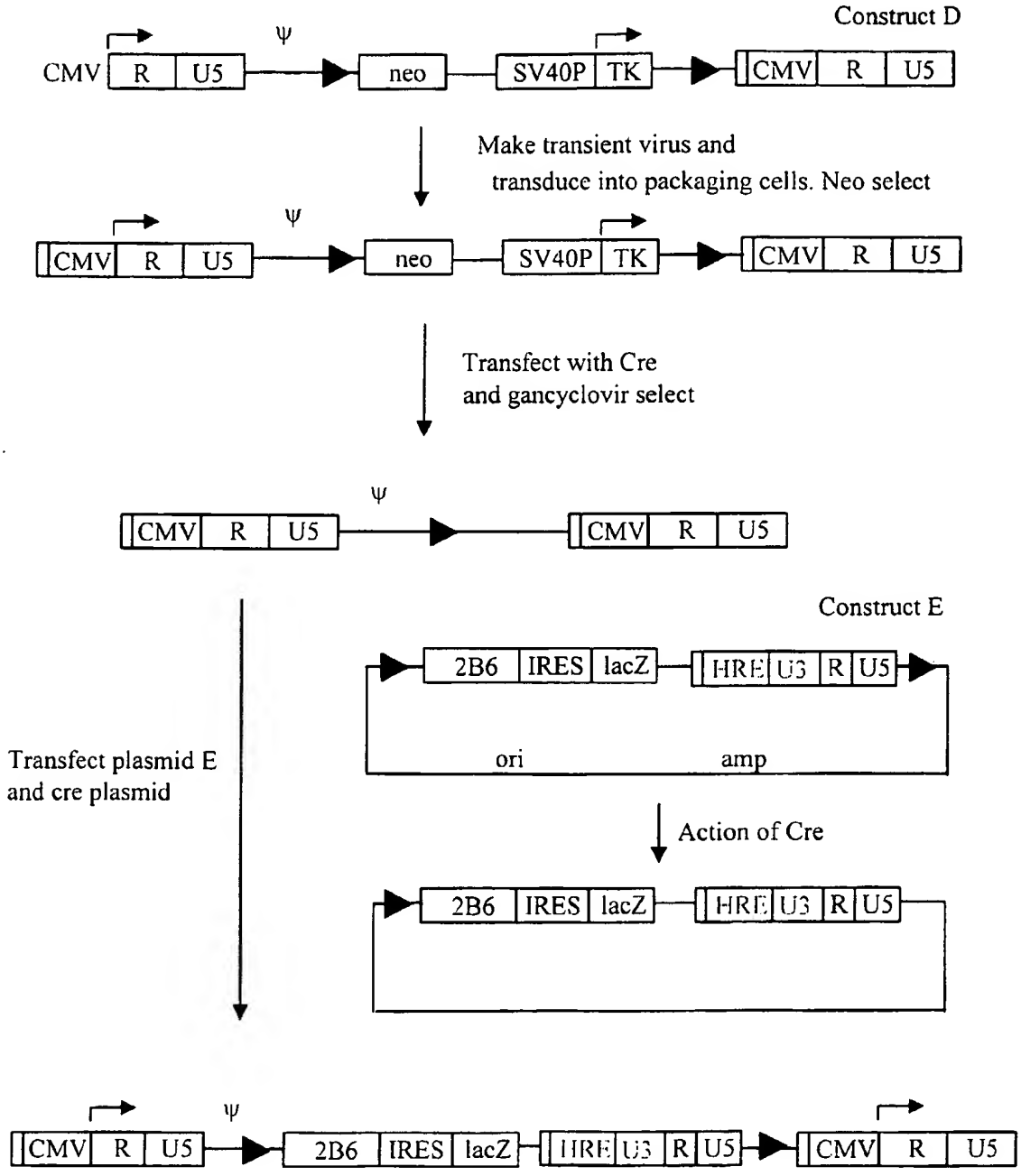


FIG. 5

MLV-based transduction for SIN vector production using Cre/loxP system

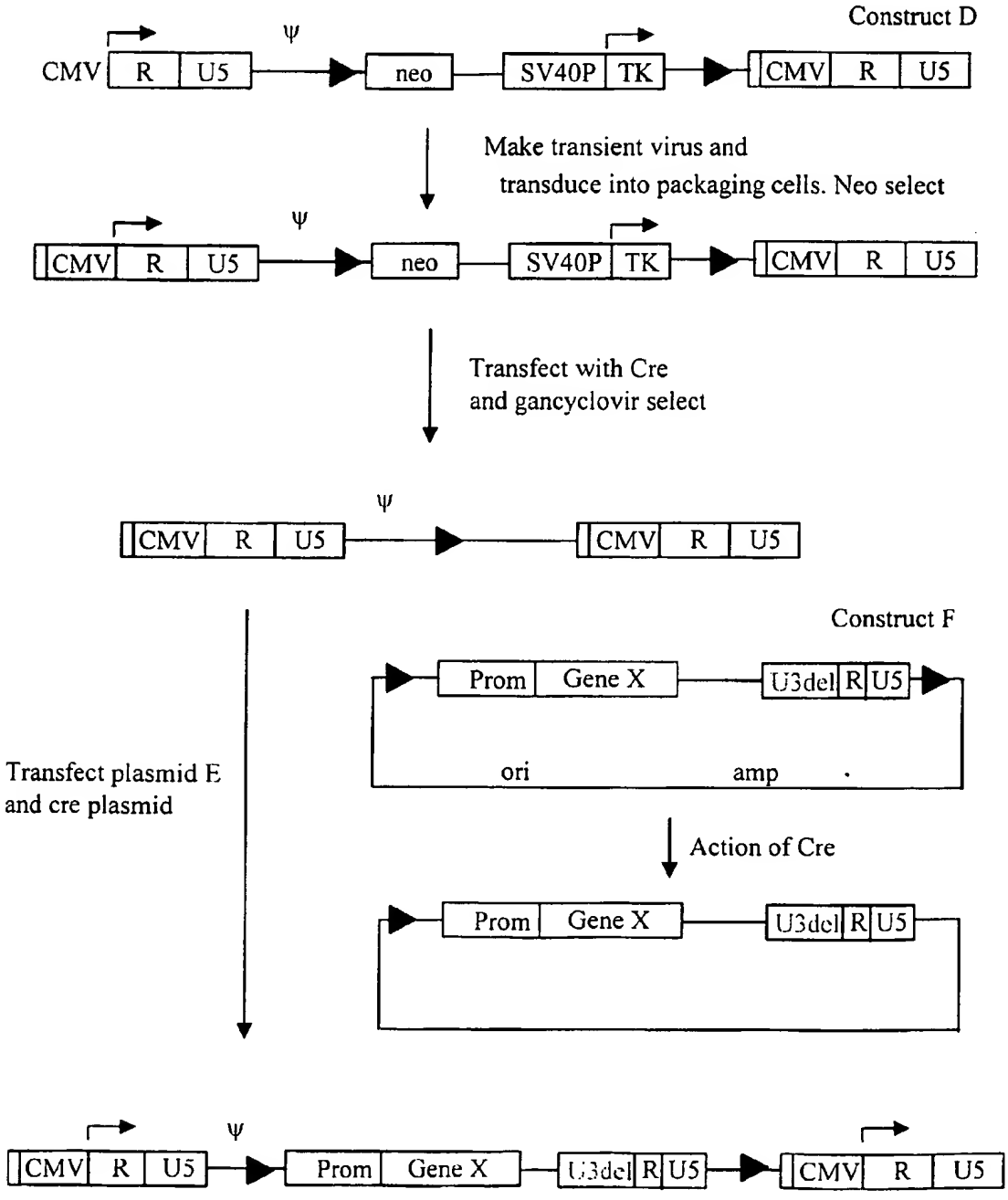


FIG. 6

MLV SIN-vector based transduction system. This general approach can be used with EIAV, HIV or MLV genomes

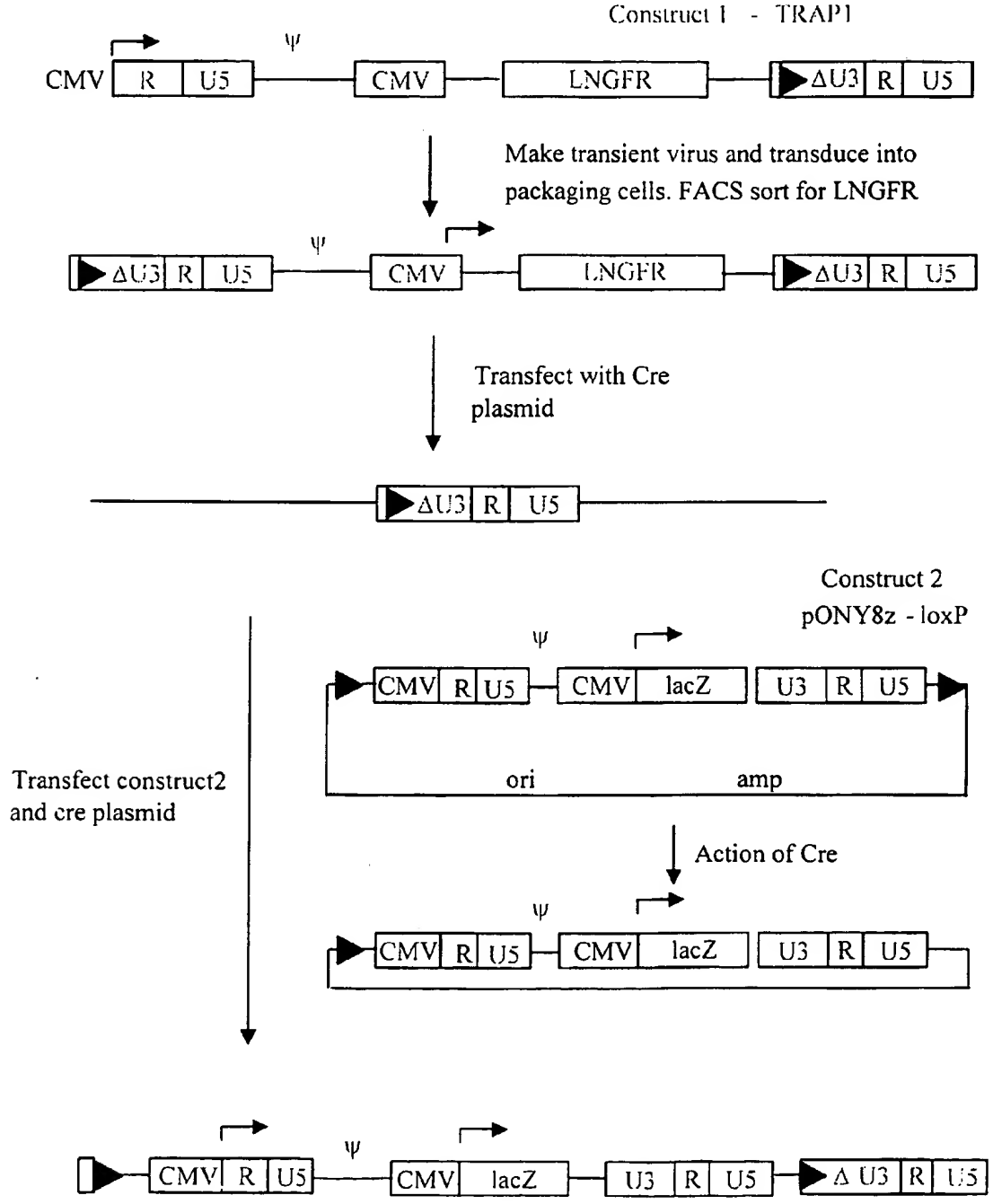


FIG. 7

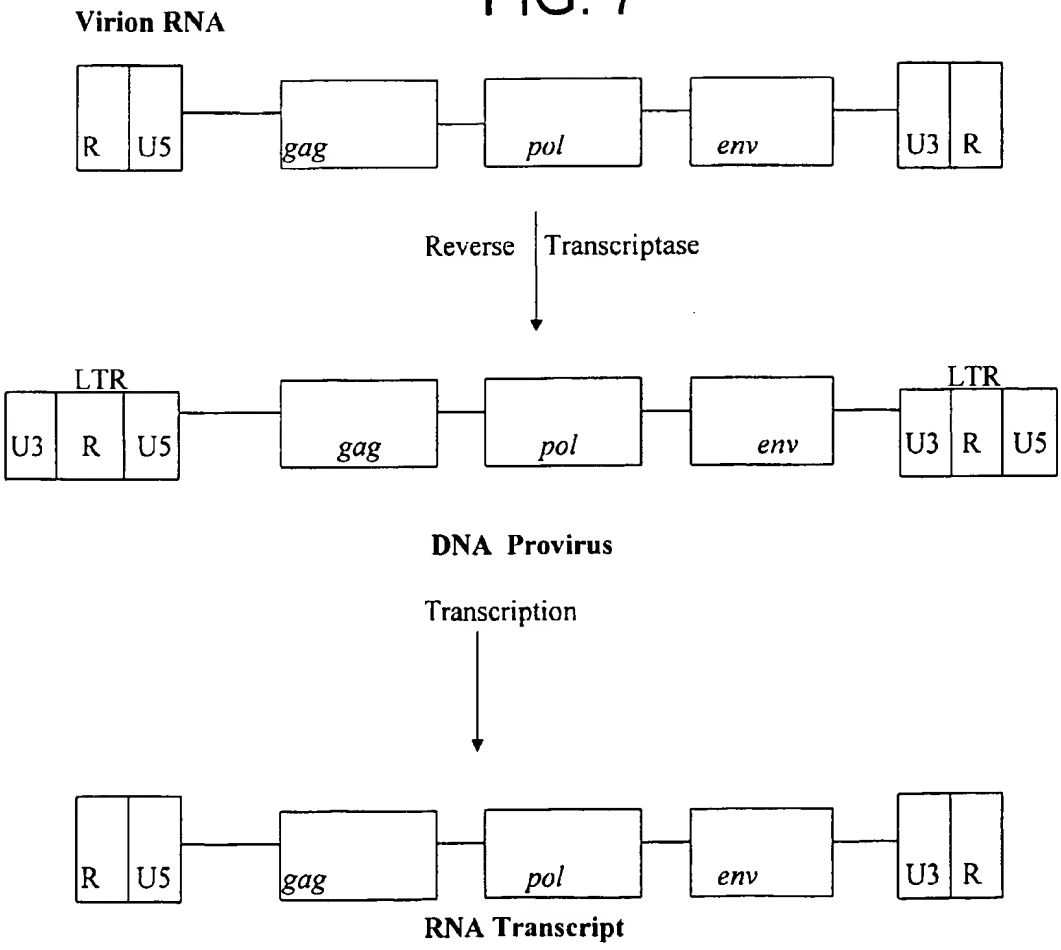

Virion RNA
R
U5
gag
pol
env
U3
R
Reverse Transcriptase
LTR
U3
R
U5
gag
pol
env
LTR
U3
R
U5
DNA Provirus
Transcription
R
U5
gag
pol
env
U3
R
RNA Transcript

FIG. 8

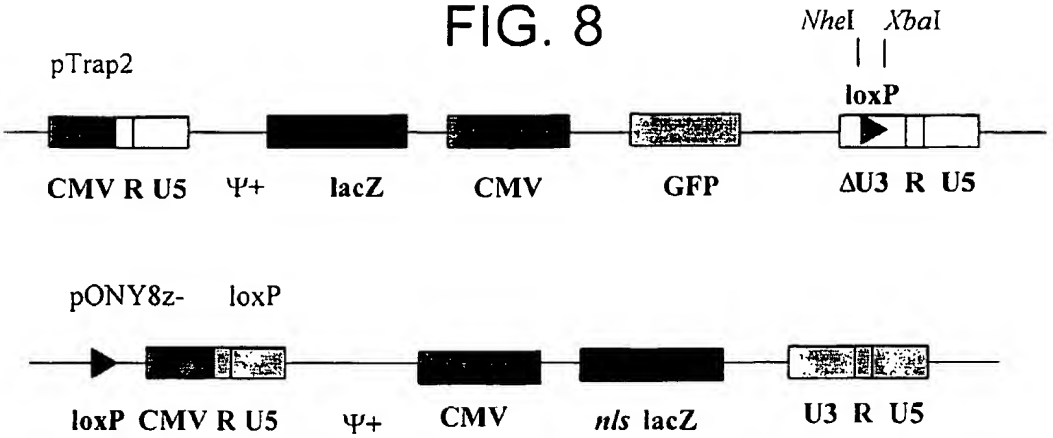
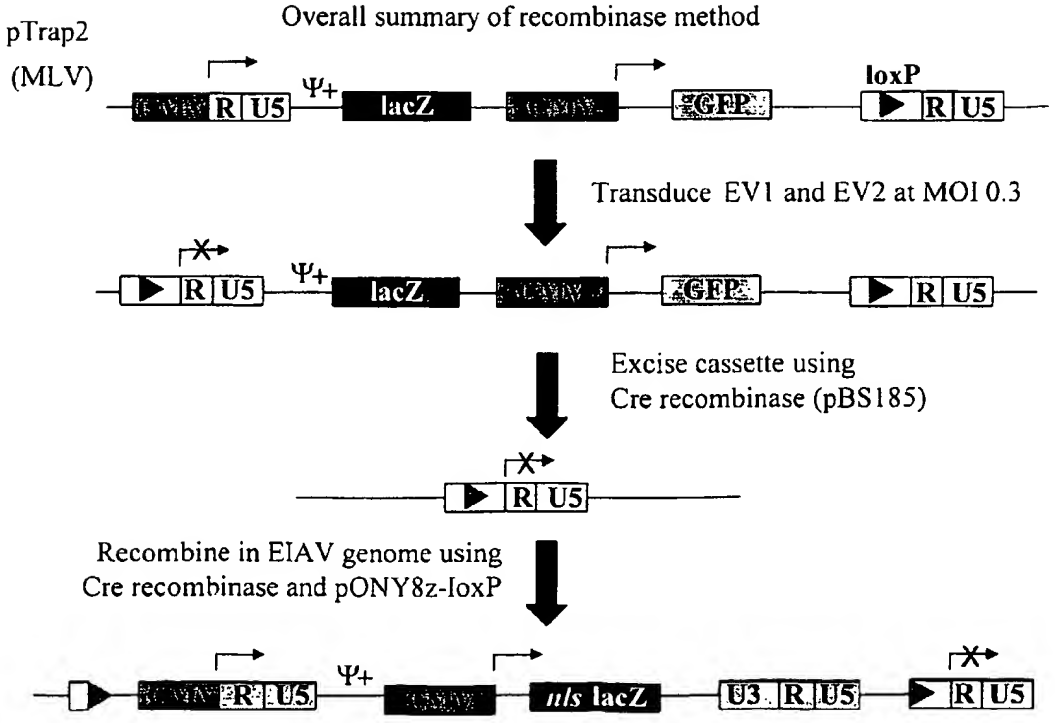

pTrap2
NheI XbaI
loxP
CMV R U5
Ψ+
lacZ
CMV
GFP
ΔU3 R U5
pONY8z- loxP
loxP CMV R U5
Ψ+
CMV
nls lacZ
U3 R U5

FIG. 9


Overall summary of recombinase method
pTrap2
(MLV)
Ψ+
lacZ
GFP
loxP
R U5
Transduce EV1 and EV2 at MOI 0.3
Excise cassette using
Cre recombinase (pBS185)
Recombine in EIAV genome using
Cre recombinase and pONY8z-IoxP
nls lacZ
U3 R U5

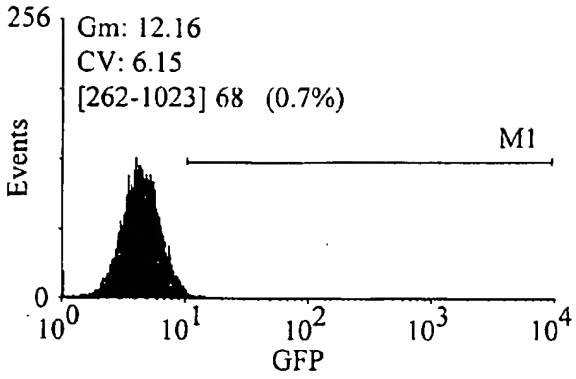


FIG. 10a

FACS analysis of EV1 packaging cells prior to transduction with Trap2 vector

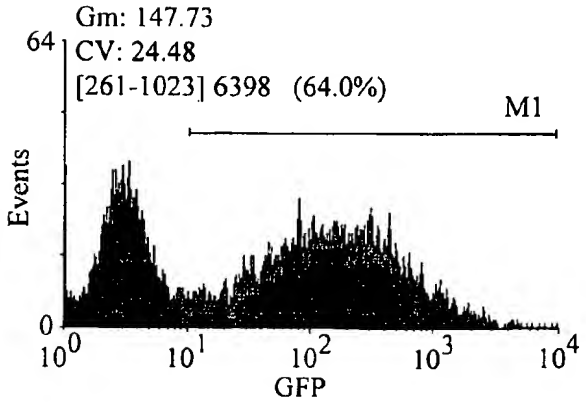


FIG. 10b

FACS analysis of EV1 packaging cell line transduced with Trap2 at an MOI of 0.3. A 5% top-slice of the highest expressers was carried out

FIG. 11

Validation of the ΔΔCt method for quantitation of GFP mRNA, relative to β-actin. A titration of total RNA from EV1 clone A was used. The difference in Ct values between the two assays is shown on the y-axis. The magnitude of the gradient must be <0.1 for the method to be valid. The gradient is 0.077, so the method is suitable.

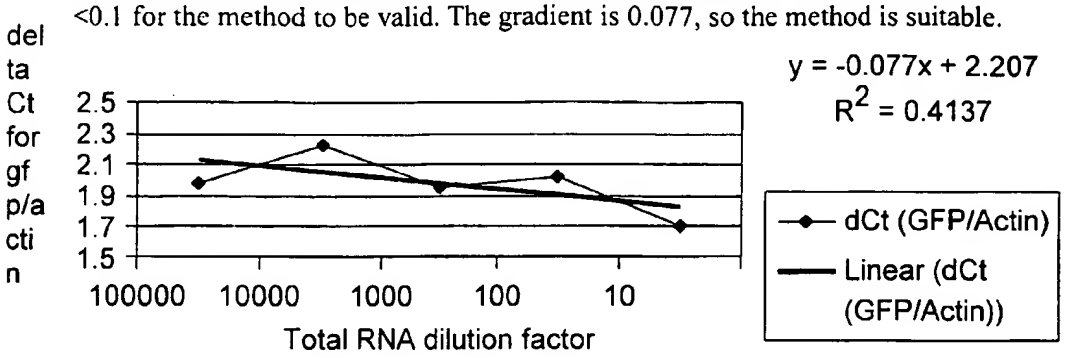


FIG. 12

Quantitation of GFP mRNA relative to control β-actin mRNA. EV2 TD cells are transduced with Trap2 at an MOI of 0.3 and are the calibrator sample with the ratio designated 1.0.

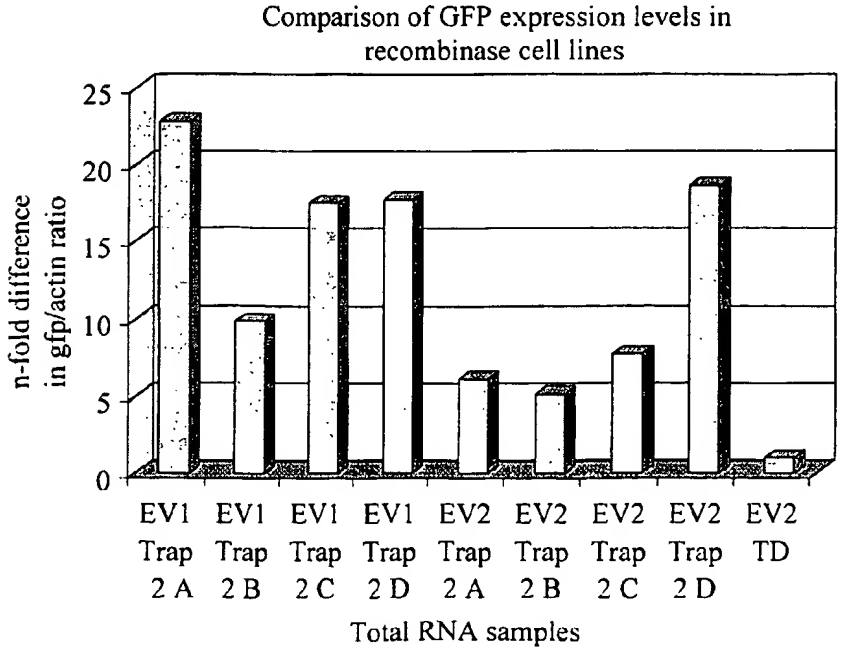
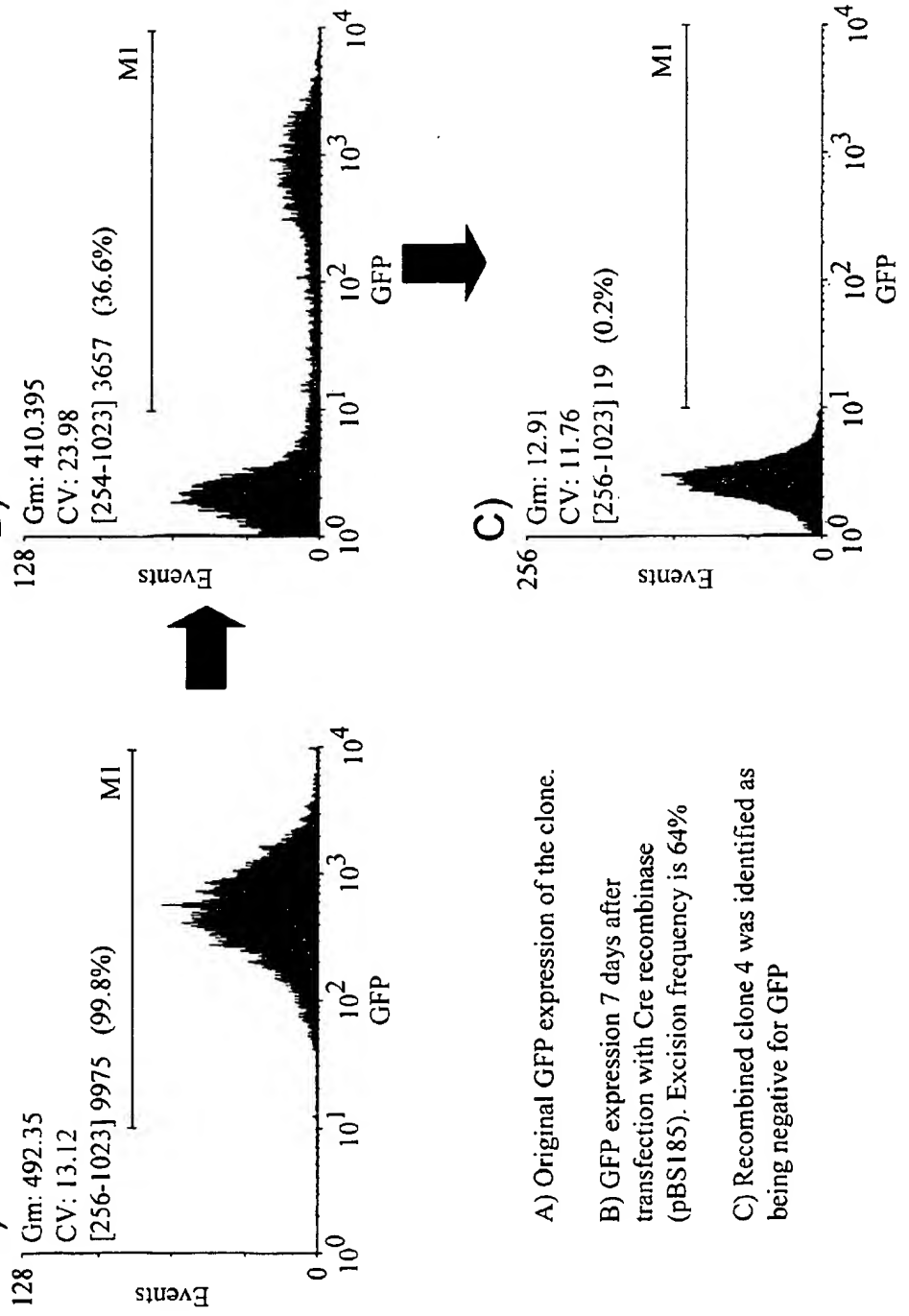


FIG. 13



A) Original GFP expression of the clone.

B) GFP expression 7 days after transfection with Cre recombinase (pBS185). Excision frequency is 64%

C) Recombined clone 4 was identified as being negative for GFP

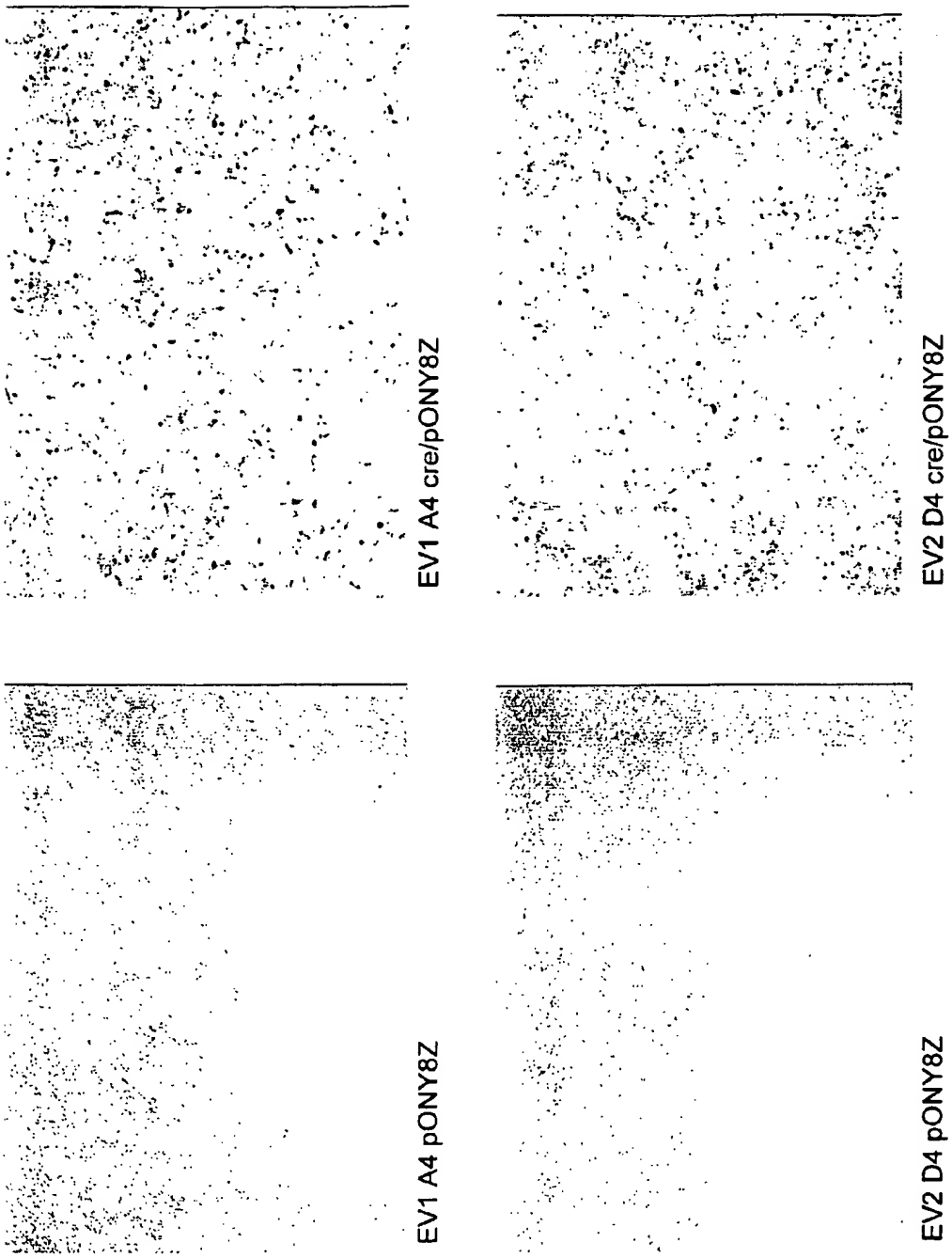

EV1 A4 cre/pONY8Z
EV2 D4 cre/pONY8Z
EV1 A4 pONY8Z
EV2 D4 pONY8Z

FIG. 14

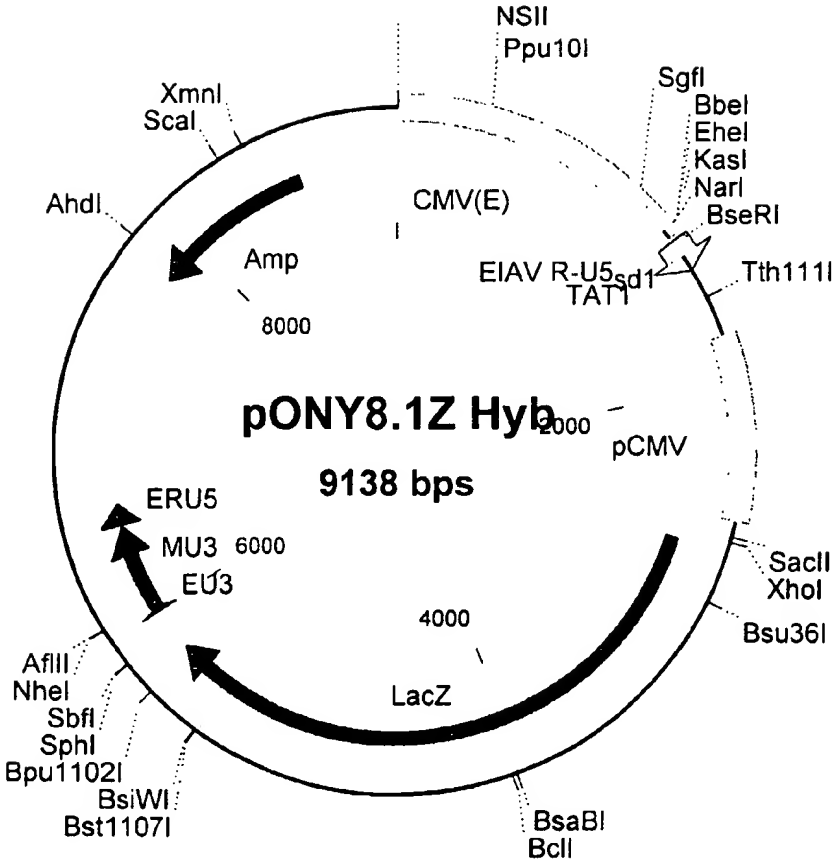

NSII
Ppu10I
SgfI
BbeI
EheI
KasI
NarI
BseRI
Tth111I
SacII
XhoI
Bsu36I
BsaBI
BclI
AflII
NheI
SbfI
SphI
Bpu1102I
BsiWI
Bst1107I
XmnI
ScaI
AhdI
CMV(E)
Amp
8000
EIAV R-U5
TAT1
sd1
pONY8.1Z Hyb
9138 bps
2000
pCMV
ERU5
MU3
EU3
6000
4000
LacZ

FIG. 15

FIG. 16

Alignment of leader and gag regions present in vectors pONY4Z, 8Z and ATG mutated 8Z vector. The later is referred to as pONY8ZA. The sequence aligned are from the NarI site in the leader to the XbaI site between the EIAV gag sequence and the CMV promoter. Sequences in the leader are shown in italic and a space is present upstream of the position of the gag ATG.

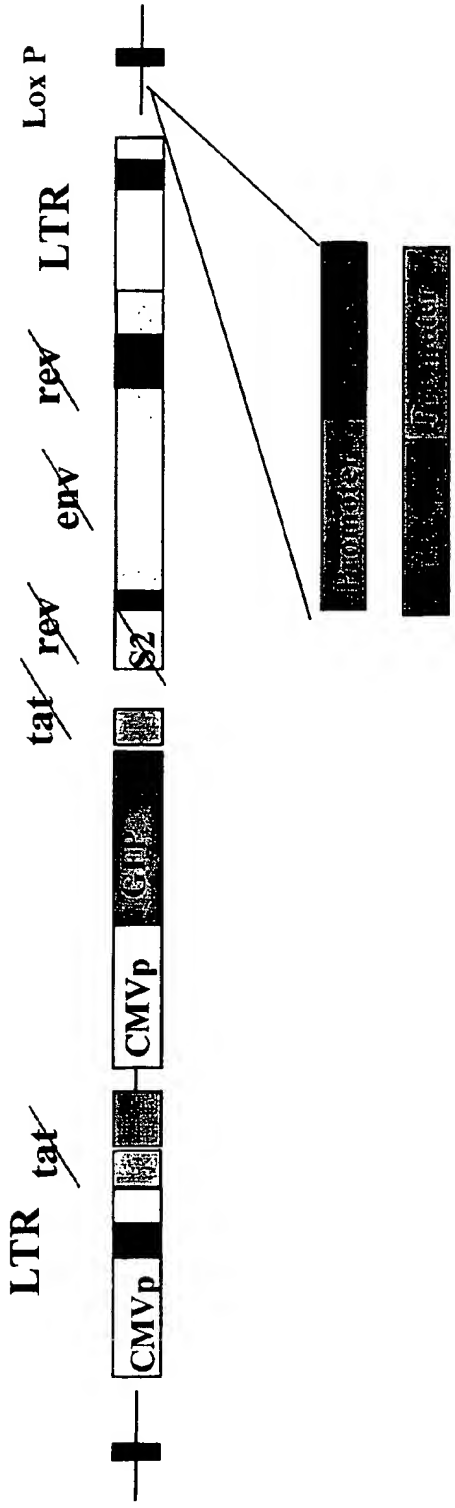
4Z	1 *cgcccgaacagggacctgagaggggcgcagaccctacctgttgaacctgg*
8Z	1 *cgcccgaacagggacctgagaggggcgcagaccctacctgttgaacctgg*
mutated 8Z	1 *cgcccgaacagggacctgagaggggcgcagaccctacctgttgaacctgg*
4Z	51 *ctgatcgtaggatccccgggacagcagaggagaacttacagaagtcttct*
8Z	51 *ctgatcgtaggatccccgggacagcagaggagaacttacagaagtcttct*
mutated 8Z	51 *ctgatcgtaggatccccgggacagcagaggagaacttacagaagtcttct*
4Z	101 *ggaggtgttcctggccagaacacaggaggacaggtaag*.at-gggagaccc
8Z	101 *ggaggtgttcctggccagaacacaggaggacaggtaag*.attgggagaccc
mutated 8Z	101 *ggaggtgttcctggccagaacacaggaggacaggtaag*.attgggagaccc
4Z	150 tttgacat-ggagcaaggcgctcaagaagttagagaaggtgacggtacaa
8Z	151 tttgacattggagcaaggcgctcaagaagttagagaaggtgacggtacaa
mutated 8Z	151 tttgacattggagcaaggcgctcaagaagttagagaaggtgacggtacaa
4Z	199 gggtctcagaaattaactactggtaactgtaattgggcgctaagtctagt
8Z	201 gggtctcagaaattaactactggtaactgtaattgggcgctaagtctagt
mutated 8Z	201 gggtctcagaaattaactactggtaactgtaattgggcgctaagtctagt
4Z	249 agacttatttcat-gataccaactttgtaaaagaaaaggactggcagctg
8Z	251 agacttatttcat-gataccaactttgtaaaagaaaaggactggcagctg
mutated 8Z	251 agacttatttcattgataccaactttgtaaaagaaaaggactggcagctg

4Z	298	agggat-gtcattccattgctggaagat-gtaactcagacgctgtcagga
8Z	300	agggat-gtcattccattgctggaagat-gtaactcagacgctgtcagga
mutated 8Z	301	agggattgtcattccattgctggaagattgtaactcagacgctgtcagga
4Z	346	caagaaagagaggcctttgaaagaacat-ggtgggcaatttctgctgtaa
8Z	348	caagaaagagaggcctttgaaagaacat-ggtgggcaatttctgctgtaa
mutated 8Z	351	caagaaagagaggcctttgaaagaacattggtgggcaatttctgctgtaa
4Z	395	agat-gggcctccagattaataat-gtagtagat-ggaaaggcatcattc
8Z	397	agat-gggcctccagattaataat-gtagtagat-ggaaaggcatcattc
mutated 8Z	401	agattgggcctccagattaataattgtagtagattggaaaggcatcattc
4Z	442	cagctcctaagagcgaaatat-gaaaagaagactgctaataaaaagcagt
8Z	444	cagctcctaagagcgaaatat-gaaaagaagactgctaataaaaagcagt
mutated 8Z	451	cagctcctaagagcgaaatattgaaaagaagactgctaataaaaagcagt
4Z	491	ctgagccctctgaagaatatct
8Z	493	ctgagccctctgaagaatatct
mutated 8Z	501	ctgagccctctgaagaatatct

FIG. 16 CONT'D

FIG. 17

Schematic representation of the structure of pONY 8.3G +/- vector genome plasmids



SEQUENCE LISTING PART OF THE DESCRIPTION

pONY8.1Z MLVHyb (SEQ ID NO 10)

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ATAGTGGTGTTTATCGCCGATAGAGATGGCGATATTGGAAAAATTGATATTTGAAAATATGGCATATTGAAAATGTCGC
CGATGTGAGTTTCTGTGTAACTGATATCGCCATTTTTCCAAAAGTGATTTTTGGGCATACGCGATATCTGGCGATAGCGC
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TATAGGTGACAGACGATATGAGGCTATATCGCCGATAGAGGCGACATCAAGCTGGCACATGGCCAATGCATATCGATC
TATACATTGAATCAATATTGGCCATTAGCCATATTATTCATTGGTTATATAGCATAAATCAATATTGGCTATTGGCCATT
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GACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTACATAACTTACGG
TAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCC
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ATGCCAAGTCCGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGACCTTACGGG
ACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACACCAATGGG
CGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAA
ATCAACGGGACTTTCCAAAATGTCGTAACAACTGCGATCGCCCGCCCCGTTGACGCAAATGGGCGGTAGGCGTGTACGG
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GAAAAGGCCTTTGTAATAAATATAATTCTCTACTCAGTCCCTGTCTCTAGTTTGTCTGTTCGAGATCCTACAGTTGGCGC
CCGAACAGGGACCTGAGAGGGGCGCAGACCCTACCTGTTGAACCTGGCTGATCGTAGGATCCCCGGGACAGCAGAGGA
GAACTTACAGAAGTCTTCTGGAGGTGTTCCTGGCCAGAACACAGGAGGACAGGTAAGATTGGGAGACCCTTTGACATT
GGAGCAAGGCGCTCAAGAAGTTAGAGAAGGTGACGGTACAAGGGTCTCAGAAATTAACTACTGGTAACTGTAATTGGG
CGCTAAGTCTAGTAGACTTATTTCATGATACCAACTTTGTAAAAGAAAAGGACTGGCAGCTGAGGGATGTCATTCCATT
GCTGGAAGATGTAACTCAGACGCTGTCAGGACAAGAAAGAGAGGCCTTTGAAAGAACATGGTGGGCAATTTCTGCTGT
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TGACCTCCATAGAAGACACCGGGACCGATCCAGCCTCCGCGGCCCCAAGCTTCAGCTGCTCGAGGATCTGCGGATCCGG
GGAATTCCCCAGTCTCAGGATCCACCATGGGGGATCCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTAC
CCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCC
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TTTATGGCAGGGTGAAACGCAGGTCGCCAGCGGCACCGCGCCTTTCGGCGGTGAAATTATCGATGAGCGTGGTGGTTAT

GCCGATCGCGTCACACTACGTCTGAACGTCGAAAACCCGAAACTGTGGAGCGCCGAAATCCCGAATCTCTATCGTGCGG
TGGTTGAACTGCACACCGCCGACGGCACGCTGATTGAAGCAGAAGCCTGCGATGTCGGTTTCCGCGAGGTGCGGATTGA
AAATGGTCTGCTGCTGCTGAACGGCAAGCCGTTGCTGATTCGAGGCGTTAACCGTCACGAGCATCATCCTCTGCATGGT
CAGGTCATGGATGAGCAGACGATGGTGCAGGATATCCTGCTGATGAAGCAGAACAACTTTAACGCCGTGCGCTGTTCGC
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GGTGCAGCGCGATCGTAATCACCCGAGTGTGATCATCTGGTCGCTGGGGAATGAATCAGGCCACGGCGCTAATCACGA
CGCGCTGTATCGCTGGATCAAATCTGTCGATCCTTCCCGCCCGGTGCAGTATGAAGGCGGCGGAGCCGACACCACGGCC
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PEsynGP (SEQ ID No 53)

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PESDSYNGP (SEQ ID No 54)

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MLV construct CZCG (SEQ ID No 55)

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PCGCLNGFR (SEQ ID No 57)

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CGCCCGGGTACCCGTGTATCCAATAAACCCTCTTGCAGTTGCATCCGACTTGTGGTCTCG
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CGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCGCAGCCATGACCCAGTCACGTAGCGATAG
CGGAGTGTATACTGGCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCAT
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AGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAAC
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TTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATC
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TGATGTTATGCGCCTGCGTCTGTACTAGTTAGCTAACTAGCTCTGTATCTGGCGGACCCG
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PLTRloxP (SEQ ID No 58)

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TCGCTTCTCGCTTCTGTTCGCGCGCTTCTGCTCCCCGAGCTCAATAAAAGAGCCCACAAC
CCCTCACTCGGGGCGCCAGTCCTCCGATTGACTGAGTCGCCCGGGTACCCGTGTATCCAA
TAAACCCTCTTGCAGTTGCATCCGACTTGTGGTCTCGCTGTTCCTTGGGAGGGTCTCCTC
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GACCCCTGCCCAGGGACCACCGACCCACCACCGGGAGGTAAGCTGGCTGCCTCGCGCGTT
TCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTC
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ATGCGTAAGGAGAAAATACCGCATCAGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCT
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GCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATA
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CGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTG
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CGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAG
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GTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCAC
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TTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACC
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CATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAA
ACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCAT
TATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGCCCTTTCGTCTTCAAGA
ATTCATACCAGATCACCGAAAACTGTCCTCCAAATGTGTCCCCCTCACACTCCCAAATTC
GCGGGCTTCTGCCTCTTAGACCACTCTACCCTATTCCCCACACTCACCGGAGCCAAAGCC
GCGGCCCTTCCGTTTCTTTGCTTTTGAAAGACCCCACCCGTAGGTGGCAA

LTR plasmid (SEQ ID No 59)

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GGTAAGCAGTTCCTGCCCCGGCTCAGGGCCAAGAACAGATGGAACAGCTGAATATGGGCC
AAACAGGATATCTGTGGTAAGCAGTTCCTGCCCCGGCTCAGGGCCAAGAACAGATGGTCC
CCAGATGCGGTCCAGCCCTCAGCAGTTTCTAGAGAACCATCAGATGTTTCCAGGGTGCCC
CAAGGACCTGAAATGACCCTGTGCCTTATTTGAACTAACCAATCAGTTCGCTTCTCGCTT
CTGTTCGCGCGCTTCTGCTCCCCGAGCTCAATAAAAGAGCCCACAACCCCTCACTCGGGG
CGCCAGTCCTCCGATTGACTGAGTCGCCCGGGTACCCGTGTATCCAATAAACCCTCTTGC
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CGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCC
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GCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAA
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GTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCC
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CAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAG
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GTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTC
ATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCT
GTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGC
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TCTTAGACCACTCTACCCTATTCCCCACACTCACCGGAGCCAAAGCCGCGGCCCTTCCGT
TTCTTTGCTTTTGAAAGACCCCACCCGTAGGTGGCAA

INTERNATIONAL SEARCH REPORT

	nal Application No
	PCT/GB 00/03837

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/867 C12N15/90 C12N5/10 C12N7/01 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, MEDLINE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	VANIN E. F. ET AL.: "Development of high-titer retroviral producer cell lines by using Cre-mediated recombination." JOURNAL OF VIROLOGY, vol. 71, no. 10, 1997, pages 7820-7826, XP002161355 ISSN: 0022-538X cited in the application the whole document --- -/--	1-51

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 February 2001	13/03/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mandl, B

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Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	KARREMAN S. ET AL.: "ON THE USE OF DOUBLE FLP RECOGNITION TARGETS (FRTS) IN THE LTR OF RETROVIRUSES FOR THE CONSTRUCTION OF HIGH PRODUCER CELL LINES" NUCLEIC ACIDS RESEARCH, vol. 24, no. 9, 1 May 1996 (1996-05-01), pages 1616-1624, XP000616161 ISSN: 0305-1048 cited in the application the whole document	1-51
A	IWAKUMA T. ET AL.: "SELF-INACTIVATING LENITVIRAL VECTORS WITH U3 AND U5 MODIFICATIONS" VIROLOGY, vol. 261, no. 1, 15 August 1999 (1999-08-15), pages 120-132, XP000882897 ISSN: 0042-6822 the whole document	1-51
A	BOAST K. ET AL.: "CHARACTERIZATION OF PHYSIOLOGICALLY REGULATED VECTORS OF THE TREATMENT OF ISCHEMIC DISEASE" HUMAN GENE THERAPY, vol. 10, no. 13, 1 September 1999 (1999-09-01), pages 2197-2208, XP000876772 ISSN: 1043-0342 the whole document	1-51

1

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 45-48

Said claims 45-48 could not searched because they were not present in the application.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.